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Aerenchyma Development in the Freshwater Marsh Species Sagittaria Lancifolia L.

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AERENCHYMA DEVELOPMENT IN THE FRESHWATER MARSH SPECIES *SAGITTARIA LANCIFOLIA* L.

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LIST OF ABBREVIATIONS

CMT	cortical microtubules
FITC	fluorescein-5-isothiocyanate
HR	hypersensitive response
PCD	programmed cell death
SE	standard error
TEM	transmission electron microscopy
TUNEL	TdT-mediated nick end labeling

ABSTRACT

Aerenchyma tissue consists of gas channels that provide a pathway for O_2 transport to waterlogged roots. Aerenchyma gas spaces are formed by cell separation or cell lysis during development. Light microscopy was used to determine how aerenchyma develops in *Sagittaria lancifolia*, a freshwater marsh species, when plants were grown in hydroponic solution. Petiole aerenchyma was formed by cell separation caused by differential cell expansion and division. In the root cortex, certain radial files of cells separated slightly from adjacent files and then lysed to form gas spaces. Development of root aerenchyma was characterized in *S. lancifolia*, *Zea mays*, and *Oryza sativa* grown at normal and low O_2 concentrations in the root zone. Root aerenchyma was formed at both O_2 concentrations in *S. lancifolia* and *O. sativa*, but only at low O_2 in *Z. mays*. The relative volume of root aerenchyma increased in *S. lancifolia* at low O_2, but not in *O. sativa* at low O_2. In *S. lancifolia*, the initial changes in root cortex cells undergoing lysis were fragmentation and condensation of the nucleus as observed by transmission electron microscopy. Breakdown of cytoplasmic organelles and disruption of the plasma membrane followed, but the cell wall remained intact. Cortex cells of *Z. mays* and *O. sativa* roots appeared similar to those in *S. lancifolia* at late stages of cell lysis, although the tonoplast in *Z. mays* cells disintegrated earlier. These observations of cell lysis were consistent with published descriptions of programmed cell death. Cortical microtubule (CMT) arrays in root cortex cells of *S. lancifolia* were oriented as expected, except in radial layers of cells termed diaphragms, where CMT arrays were parallel to the root axis. This orientation would lead to the radial expansion of these cells and could produce the separation of the files of cortex

cells that precedes cell lysis. It is concluded that there are multiple mechanisms of aerenchyma formation. Three mechanisms of root lysis found in these studies are: constitutive cell lysis (*O. sativa*), induction of lysis by low O_2 (*Z. mays*), and both constitutive cell lysis and induction of cell lysis by low O_2 (*S. lancifolia*).

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The development of aerenchyma tissue is an important adaptation for plants that live in waterlogged conditions. Waterlogged soils quickly become hypoxic and this reduces plant growth. Aerenchyma tissue allows the internal transport of O_2 from the shoots to the hypoxic root zone, where it is consumed in cellular respiration and oxidation of the soil surrounding the roots (Armstrong, 1979; Crawford, 1982). By providing aeration in a hypoxic environment, aerenchyma allows marsh species to maintain high productivity in an otherwise unproductive habitat.

Knowledge gained by studying the development of aerenchyma tissue may have implications for other developmental processes. Aerenchyma formation involves cell separation and cell death, both of which are active in many other plant developmental changes such as formation of the spongy mesophyll or the abscission zone. A specialized type of cell death called "programmed cell death" has been the recent subject of intense research and has been proposed to be active in aerenchyma development as well as many other types of plant cell death.

The main objective of my research has been to investigate aerenchyma development in the freshwater marsh species *Sagittaria lancifolia*. Three specific aspects of aerenchyma development were addressed: anatomical development of the root and petiole, environmental influence on the formation of the tissue, and characterization of the cell death that forms the root tissue. Aerenchyma development by cell lysis in this marsh

species was then compared with aerenchyma development by cell lysis in two other species.

In this chapter I summarize previous research conducted on aerenchyma tissue, outline some of the major unanswered questions in the field, and detail the specific objectives of my research. In Chapter 2, I present results of a microscopy study of the development of the aerenchyma in the root and petiole in *S. lancifolia* grown in hydroponic solution. Chapter 3 analyzes the effect of O_2 and ethylene concentrations on the induction of aerenchyma, the relative volume of aerenchyma produced, and the activity of cellulase in *S. lancifolia*. The development of root aerenchyma at different O_2 concentrations in *Zea mays* and *Oryza sativa* is compared to the *S. lancifolia* response. Chapter 4 characterizes the changes in root cells undergoing cell lysis to determine if the lytic process is an example of a type of programmed cell death. The arrangement of cortical microtubules was examined to determine if changes in microtubule orientation relate to cell lysis and aerenchyma development. Chapter 5 presents the conclusions of this research.

LITERATURE REVIEW

Marsh species generally live in waterlogged soils that are characterized by very low O_2 concentrations. Plants living in these conditions possess a number of anatomical and metabolic adaptations that permit survival at low O_2 concentrations and occasional flooding (Vartapetian and Jackson, 1997). An pervasive anatomical adaptation in marsh species is the formation of aerenchyma tissue. The development of this tissue is almost universal among marsh species growing under all conditions (constitutive production).

Anatomy of Aerenchyma

Aerenchyma tissue is composed of interconnected gas spaces in the root and shoot cortex of a plant (Armstrong and Armstrong, 1988). The individual gas spaces are called lacunae, and the gas spaces together are called aerenchyma tissue. Aerenchyma gas spaces can be bisected (perpendicular to the axis of the root or shoot) at regular intervals by porous layers of diaphragm cells (Kaul, 1971; 1973; 1974). These layers add support to the plant while allowing gases to travel between gas spaces. Very little is known about the physiological function of these cells, however.

Aerenchyma is classified into two types based on how gas spaces form (Justin and Armstrong, 1987). Gas spaces in "schizogenous" aerenchyma are formed by cell separation, while gas spaces in "lysigenous" aerenchyma are formed by cell lysis. Classification is usually determined by examining whether gas spaces of mature tissue are surrounded by intact (formed by cell separation) or lysed (formed by cell lysis) cells, rather than by examination of how the tissue develops (Smirnoff and Crawford, 1983).

The gas space volume relative to the entire volume of the organ (or relative volume of aerenchyma) varies, both in plant roots and shoots. In *Oryza sativa* L. cv. RB3, aerenchyma gas spaces can occupy 60-70% of the total volume of adventitious roots (Jackson, Fenning, and Jenkins, 1985). In a different variety of *O. sativa* (cv. Colusa), however, only 15% of the relative volume of the roots is aerenchyma (Varade, Letey, and Stolzy, 1971). This suggests there can be variability in relative aerenchyma volume even among varieties of the same species. *Rumex maritimus*, a flood-tolerant plant, has root aerenchyma formed by cell separation that occupies 30% of its root volume (Laan et al., 1989). In *Spartina patens*, aerenchyma gas spaces occupy up to 50% of the root volume

(Burdick and Mendelssohn, 1990). A study of aerenchyma volumes in *Spartina alterniflora* found that the short form of this species has 3.1%, 23%, 36.1%, and 29% relative volume of aerenchyma in its leaves, stem bases, rhizome, and roots, respectively, compared to 5%, 41.8%, 45.3%, and 28.8% in the tall form (Arenovski and Howes, 1992).

There are still many unanswered questions about the anatomy of this tissue. For instance, the relative number of species that form aerenchyma by cell lysis versus cell separation (or both) is unknown. It appears that cell lysis occurs mainly in root aerenchyma development, and cell separation in shoot aerenchyma development, but these generalizations have rarely been tested. It is also not known if the type of formation (cell lysis or separation) can be correlated with the relative volume of aerenchyma. Finally, it is not clear if there are different relative volumes of aerenchyma in roots and shoots among species.

Environmental Influences on Aerenchyma Formation

In some species, the induction of gas space formation and/or relative volume of gas spaces can be influenced by O_2 concentration in the root zone. In *Helianthus annuus* (Kawase and Whitmoyer, 1980), *Lycopersicon esculentum* (Kawase and Whitmoyer, 1980), *Triticum aestivum* (Varade, Stolzy, and Letey, 1970; Huang et al., 1994), and *Zea mays* (Drew, Jackson, and Giffard, 1979), hypoxic conditions induce the formation of aerenchyma where there normally would be none. Relative root aerenchyma volume increases as root zone O_2 concentration decreases in *O. sativa* (Das and Jat, 1977; Justin and Armstrong, 1991; Kludze, DeLaune, and Patrick, 1993) and *S. patens* (Burdick, 1989), although induction of aerenchyma formation is not affected by O_2 in these species (or varieties).

Environmental stresses other than hypoxia may also effect aerenchyma development in some species. In *Nardus stricta*, relative aerenchyma volume increases as growth nutrient concentration decreases (Smirnoff and Crawford, 1983), and relative aerenchyma volume of the root increases in *Z. mays* as nitrogen concentration decreases (Konings and Verschuren, 1980). Nitrogen or phosphorus deficiency induces root aerenchyma formation in *Z. mays* (Drew, He, and Morgan, 1989). In *T. aestivum*, high temperature (42 °C) (Sojka, Joseph, and Stolzy, 1972) and high light intensity (3500 ft candles vs. 700 ft candles) (Luxmoore, Sojka, and Stolzy, 1972) result in a greater relative volume of root aerenchyma than the volume produced by aeration stress. High temperature and high light intensity also produce a greater relative volume of root aerenchyma in *O. sativa* (Varade, Letey, and Stolzy, 1971).

However, in some species aerenchyma formation is unaffected by environmental conditions. In *O. sativa* cv. RB3 (Jackson, Fenning, and Jenkins, 1985) and *O. sativa* cv. Colusa (Varade, Letey, and Stolzy, 1971), relative aerenchyma volume is constant for plants grown in different aeration treatments. *Sagittaria alterniflora* has the same relative volume of aerenchyma when grown in flooded or drained conditions (Arenovski and Howes, 1992). Examination of aerenchyma production in nine British flood-tolerant species found that five species did not change their relative aerenchyma volumes when grown in different flooding regimes (Smirnoff and Crawford, 1983). Therefore, there is constitutive (rather than environmental) control of aerenchyma development and relative volume in some species.

There is still much to learn regarding the influence of the environment, or lack thereof, on aerenchyma development. Whether all marsh species, or species adapted to

waterlogged soils, form aerenchyma constitutively is not known; nor is the amount of environmental control over the relative volume of gas spaces. All agronomic species that produce aerenchyma appear to do so strictly as a response to a change in the environment, but there has been little investigation of aerenchyma production under field (rather than laboratory) conditions. Also of interest is which environmental factors control the production of aerenchyma in these species.

Function of Aerenchyma

Aerenchyma gas spaces serve as a pathway for O_2 from the shoots to the roots in a hypoxic environment. The pathway they provide is continuous and bi-directionally transports not only O_2, but other gases. Gases move by diffusion along a concentration gradient and, in some species, by mass flow along a pressure gradient (Dacey, 1980; Grosse and Mevi-Schutz, 1987; Brix, 1988). Oxygen from the aerenchyma aerates the belowground portion of the plant to maintain aerobic cellular respiration of roots and rhizomes. In some cases, this O_2 can also diffuse out of the root and oxidize soil compounds produced under anoxic conditions (including reduced iron, manganese, and hydrogen sulfide) which can be toxic to plants (Gambrell, DeLaune, and Patrick, 1991).

The importance of aerenchyma as a source of O_2 to the roots and surrounding root zone has been evaluated in a number of studies. Oxygen release from the roots of plants (Armstrong and Armstrong, 1988) and increases in the redox potential of the soil surrounding plants (Justin and Armstrong, 1987) are consistent with the transport through the aerenchyma and diffusion into the soil of significant amounts of O_2. In *Phragmites australis* (Brix, 1989) and five other marsh species (Bedford, Bouldin, and Beliveau, 1991) most of the O_2 transported through the aerenchyma to the roots is consumed by cell

respiration, causing limited root zone oxygenation. Under anaerobic conditions, *Z. mays* roots with aerenchyma have higher ATP/ADP ratios than *Z. mays* roots lacking aerenchyma (Drew, Saglio, and Pradet, 1985), suggesting aerenchyma O_2 transport is helpful in maintaining aerobic respiration. In *S. patens*, the amount of O_2 transported through the aerenchyma is not sufficient to fully support aerobic metabolism (Burdick and Mendelssohn, 1987; 1990).

Many studies have focused on the transport of O_2, but ignored the transport of other gases in the aerenchyma. Both CO_2 and methane can be transported in relatively high concentrations from the roots and rhizomes to the atmosphere through the aerenchyma. The CO_2 in aerenchyma can originate from respiration (Brix, 1988; 1989; Bedford, Bouldin, and Beliveau, 1991) and, in at least *O. sativa*, from soil microorganisms (Higuchi, 1982; Higuchi, Yoda, and Tensho, 1984). The transport of methane to the atmosphere, through aerenchyma and other pathways, has been reviewed (Chanton and Dacey, 1991), but the movement of CO_2 and the implications of this movement for photosynthesis has received less attention.

The existence of high CO_2 concentrations in the aerenchyma of marsh species has been known for many years, but the relationship between the high concentration and photosynthesis has only recently received attention. In *Typha latifolia*, CO_2 concentration in the aerenchyma is always higher than in the surrounding atmosphere, even when the concentration fluctuates diurnally and annually (Constable, Grace, and Longstreth, 1992). Laboratory studies suggest this aerenchyma CO_2 can be fixed for photosynthesis in *T. latifolia* (Constable and Longstreth, 1994). Other studies confirmed photosynthetic uptake of CO_2 from the aerenchyma of *P. australis* (Brix, 1990) and *S. alterniflora*

(Hwang and Morris, 1992) and found that the primary sites of CO_2 fixation from the aerenchyma are in the stems and leaf sheaths of these species.

Mechanism of Aerenchyma Formation

Ethylene gas is a plant hormone produced by roots under hypoxic conditions (Brailsford et al., 1993) and it is thought to be an early "signal" for the production of aerenchyma. Increases in ethylene concentration correlate with increases in the activity of cellulase, an enzyme that catalyzes the breakdown of cell walls. Cellulase activity has been hypothesized to cause the cell lysis that forms aerenchyma gas spaces (Drew, Jackson, and Giffard, 1979; Kawase, 1981; He, Drew, and Morgan, 1994). Ethylene-induced cellulase activity acts randomly on cortex cells to form aerenchyma in stems of *H. annuus* (Kawase, 1979). Links between ethylene concentration, cellulase activity, and aerenchyma production can be found in *Z. mays* (Konings, 1982; He, Drew, and Morgan, 1994), *L. esculentum*, and *Phaseolus vulgaris* (Kawase, 1981). Aerenchyma development and ethylene production is also linked in the dune-slack plant, *Scirpus americanus* (Seliskar, 1988). Recent work has linked components of an ethylene signal transduction cascade to increased cellulase activity and cell death in *Z. mays* (He, Morgan, and Drew, 1996).

Ethylene apparently may not regulate aerenchyma formation in all species, however. Unlike in *Z. mays*, the same relative volume of root aerenchyma is produced in *O. sativa* (cv. RB3) when they are grown in different concentrations of ethylene (Jackson, Fenning, and Jenkins, 1985). This indicates that there may be an ethylene-dependent and an ethylene-independent formation of aerenchyma by cell lysis.

Differences in the development of root aerenchyma by cell lysis are also supported by different cell structure changes preceding cell death in *Z. mays* and *O. sativa*. In lysing cortex cells of *Z. mays* roots, the tonoplast ruptures and the cell collapses before lysis of the cell wall (Campbell and Drew, 1983). Granularity of the vacuole, vesicles and electron-lucent regions in the cytoplasm, and retraction of the plasma membrane from the cell wall are also observed in *Z. mays* cortex cells. In *O. sativa*, the cell wall is affected before loss of tonoplast integrity (Webb and Jackson, 1986). Cortex cells in *O. sativa* also have dilation of the organelles at late stages of cell lysis, and cytoplasmic material between the retracted plasma membrane and cell wall.

Other structural components of the cell such as cortical microtubules may play a role in both cell lysis and cell separation to form aerenchyma. The orientation of cortical microtubule arrays are thought to direct cell wall placement in plant cells, which directly affects the subsequent direction of cellular growth. In *Z. mays* and *Pilea cardierei*, cortical microtubules direct the formation of localized cell wall thickenings which cause differential cell growth and formation of intercellular spaces (Galatis, 1988; Apostolakos, Galatis, and Panteris, 1991). Cortical microtubules could potentially direct cell separation to form aerenchyma as well. Hormones have been known to re-orient cortical microtubule arrays, but recent microinjection studies found that this re-orientation is much faster than previously suspected (Wymer and Lloyd, 1996). In roots, an area beyond the region of cell division called the post-mitotic isodiametric growth region is thought to be the site of the establishment of cell growth patterns in response to hormones (Baluska, Barlow, and Kubica, 1994). According to one hypothesis, the cortical microtubule orientations in this region respond to ethylene in a mechanism that causes cell lysis to form root

aerenchyma (Baluska, Barlow, and Kubica, 1994). Cells of the cortex in *Z. mays* display microtubule disorganization at lower concentrations of ethylene than surrounding cells, or are thought to be more "sensitive" to ethylene (Baluska et al., 1993). These cells with disorganized microtubules correlate with those that later lyse to form aerenchyma. The microtubule reorientations are hypothesized to weaken the cell walls, making them more susceptible to cell lysis and aerenchyma formation (Baluska et al., 1993).

There are obviously many unanswered questions about the mechanism(s) of aerenchyma formation. The possibility of two sequences leading to cell lysis, one being ethylene-dependent and the other ethylene-independent, needs to be explored. Given the constitutive nature of some cell lyses as compared to the induced nature of others, it is intriguing to speculate that there may have been evolutionary divergence in the pathway. While most of the mechanistic work has focused on ethylene and cellulase, other possibilities such as microtubules or other enzymes need to be more seriously addressed in the literature.

Programmed Cell Death and Aerenchyma

Orderly cellular death during development, such as that seen in cell lysis to form aerenchyma, is often studied as a type of programmed cell death (PCD). PCD is an active process involving new protein synthesis by a cell (in response to an external or internal signal) to cause its own death. PCD can be contrasted with necrosis, a passive process caused by an outside stimulus. In necrosis, mitochondria are the primary sites of cellular disruption, while in PCD the nucleus is affected first (Singh and Anand, 1994).

Apoptosis is a morphological description of one type of PCD and is often used incorrectly as a synonym for PCD (Schwartz et al., 1993). Apoptotic cell death is characterized by the morphological criteria of chromatin condensation (pycnosis), nuclear fragmentation, condensation of the cytoplasm, plasma membrane blebbing, and formation of apoptotic bodies (Kerr, Wyllie, and Currie, 1972; Schwartz et al., 1993). Also characteristic of apoptosis is fragmentation of the DNA into oligonucleosomal-sized fragments which yields a DNA ladder on an agarose gel. Fragmented nuclear DNA can also be identified by the TUNEL (TdT-mediated nick end labeling) assay (Gavrieli, Sherman, and Ben-Sasson, 1992), which tags free 3'-OH ends of DNA that have been cleaved by endonucleases. Cell death in which the DNA either appears not to fragment, or fragments without yielding a DNA ladder, is termed "non-apoptotic" PCD (Schwartz et al., 1993). Two non-apoptotic morphologies have been described by Clarke (1990) and include disintegration of the cell contents by lysosomes (autophagic degeneration) and disintegration by cytoplasmically-located enzymes (non-lysosomal degeneration).

Much of our knowledge about the process of PCD comes from the study of animal systems where three stages have been identified (Kroemer et al., 1995). In the induction phase, the cell cycle arrests (Chiarugi et al., 1994; Meikrantz and Schlegel, 1995) and the positive or negative expression of certain gene products (reviewed by White, 1993) determines whether the cell will undergo cell death or pass through another cell cycle. The effector phase includes the cellular events that cause cell death, such as the degradation of nuclear DNA by endonucleases (Peitsch, Mannherz, and

Tschopp, 1994) and the removal of proteins by ubiquitins (Schwartz et al., 1993). In the degradation phase, morphological features such as cytoplasmic condensation, cell shrinkage, collapse of the nucleus and cell membrane disruption occur; the latter usually occurring late in the sequence of events (Schwartz et al., 1993). After complete cellular degradation in animals, the cell remnants are engulfed by neighboring cells.

In the past two years there were five reviews of PCD in plants (Greenberg, 1996; Havel and Durzan, 1996a; Jones and Dangl, 1996; Mittler and Lam, 1996; Pennell and Lamb, 1997), indicative of recent interest in this topic. Research on PCD in plants has been mainly concerned with identifying apoptotic characteristics in plant cells undergoing cell death, so many studies have employed the TUNEL procedure. Relatively fewer have identified morphological changes that occur during cell death in plants.

The disintegration of tracheary cell contents during xylem development is one example of PCD in plants. In tracheary differentiation of *P. sativum* and *Zinnia elegans*, nuclear DNA fragmentation detectable by the TUNEL assay has been identified (Mittler and Lam, 1995a; Groover et al., 1997). Although TUNEL labeling of nuclei is characteristic of apoptosis, morphological studies of *Z. elegans* tracheary element development failed to find evidence of apoptotic morphology (Groover et al., 1997). In tracheary element differentiation in *Z. elegans*, nuclear fragmentation, as identified by DNA laddering, occurs prior to cell death and correlates with an increase in the activity of a 43-kDa enzyme thought to be a nuclease (Thelen and Northcote, 1989). The activity of this nuclease is inhibited by zinc and EDTA, like nucleases involved in animal PCD.

Other types of cell death in plants have been identified as being PCD- or apoptotic-like, as well. In a floral organ abortion mutant of *Z. mays*, the protein encoded by a gene thought to be required for cell death during sex determination has homology with hydroxysteroid dehydrogenases (DeLong, Calderon-Urrea, and Dellaporta, 1993). Since the activation of this protein appears to be critical to cell death they have argued that programmed cell death is active in this process. PCD-like death has also been identified in diploid parthenogenesis of early embryos of Norway Spruce (Durzan, 1996; Havel and Durzan, 1996b) and is assumed to occur during megasporogenesis of seed plants (Bell, 1996). In diploid parthenogenesis, structured disintegration of the nucleus is identified by observation of fragmentation (morphologically), and positive TUNEL results (biochemically) (Havel and Durzan, 1996b).

The cell death stage of the hypersensitive response (HR) in the best-studied example of PCD in plants. The HR occurs when a nonpathogen or an avirulent strain of a pathogen infects a plant (reviewed by Mittler and Lam, 1996). During the HR, cell wall cross-linking increases in the invaded cell, the invaded cell dies and surrounding cells induce defensive reactions. Reactive oxygen species are involved in this process because the entrance of the pathogen into the cell causes a corresponding increase in reactive oxygen species (Goodman, 1994; Levine et al., 1994). This "oxidative burst" mediates the production of H_2O_2 in the plant which, along with oxygen radicals, may be involved in the death of the infected cell (Levine et al., 1994). The signaling and death of some animal cells is regulated by the production of oxygen radicals, although a

recent report has questioned their role in causing the cell death (Jacobson and Raff, 1995).

Research on the HR has focused on nuclear changes that occur in association with cell death. The HR in *Vigna unguiculata* (cowpea) and *L. esculentum* involves nuclear fragmentation that appears to resemble the ladder-like fragmentation of apoptotic animal cells (Ryerson and Heath, 1996; Wang, Bostock, and Gilchrist, 1996). Fragmented (identified by TUNEL), though not laddered, DNA correlates with an increase in activity of several deoxyribonucleases in the nuclei of cells undergoing the HR (Mittler and Lam, 1995b). These nucleases were also found to be positively regulated by calcium and negatively regulated by EGTA, EDTA and zinc, as animal nucleases are. The nucleases were active late in the death process unlike the early activation in animal PCD, however (Peitsch, Mannherz, and Tschopp, 1994).

One of the major frustrations with PCD research in plant systems is the search for features that have been defined in animal systems. Whether plants actually go through apoptosis in a manner similar to animals has yet to be proven. Much of the research on plant PCD has focused only on the HR and tracheary element differentiation. The HR is an environmentally-induced cell death, however, and may be quite different from constitutive cell death in other plant processes. Other plant systems that more fully represent the variations of cell death expressed in plants need to be identified and studied.

OBJECTIVES AND APPROACHES OF RESEARCH

Sagittaria lancifolia, a freshwater marsh species, was chosen for the following studies because it is adapted to flooded conditions, can be a dominant member of the

freshwater marsh communities bordering the northern Gulf of Mexico, and possesses extensive aerenchyma. The questions I chose to address in this study were: What are the developmental patterns of the aerenchyma in the root and petiole of *S. lancifolia*? Do changes in root zone aeration affect the relative aerenchyma volume of the root and petiole in this species? Do changes in ethylene concentration in the root zone affect relative aerenchyma volume of the root and petiole? Do changes in either aeration or ethylene in the root zone affect cellulase activity in the roots or petioles? Do changes in O_2 concentration affect the development of root aerenchyma in *Zea mays* and *Oryza sativa* in the same manner as in *S. lancifolia*? Does the breakdown of cells during the formation of root aerenchyma follow a predictable pattern of changes similar to published descriptions of programmed cell death? How does the arrangement of cortical microtubules relate to the development of root aerenchyma in *S. lancifolia*?

My first objective was to examine the development of root and petiole aerenchyma in *S. lancifolia*. Based on the appearance of the mature aerenchyma tissue, I hypothesized that the root aerenchyma would develop by cell lysis and the shoot aerenchyma by cell separation. To test this hypothesis, roots and petioles were embedded in resin and sections from different developmental stages were observed with a light microscope. The results confirmed my hypothesis.

My second objective was to determine if the relative aerenchyma volume of roots and petioles in *S. lancifolia* is affected by changes in O_2 or ethylene concentration. A secondary objective was to determine if cellulase activity in the roots or petioles could be related to aerenchyma volume. I hypothesized that *S. lancifolia* root and petiole aerenchyma would produce approximately the same relative volume of aerenchyma when

grown in a changing environment. Oxygen and ethylene concentrations were manipulated at the root zone in a laboratory experiment and cross-sectional percentage of gas spaces and cellulase activity were measured. Relative aerenchyma volume in the roots increased as a result of low O_2 concentration. The process of cell lysis in response to O_2 treatment in *S. lancifolia* was also compared to that in *Z. mays* and *O. sativa*. I hypothesized that aerenchyma development and response to O_2 concentration in *O. sativa* would be more similar to *S. lancifolia* than to *Z. mays* because they both appear to produce their aerenchyma constitutively. In these experiments, both *S. lancifolia* and *O. sativa* produced aerenchyma constitutively, while formation of aerenchyma in *Z. mays* was induced at low O_2 concentration but not at normal O_2 concentration. The development of aerenchyma at different O_2 concentrations was different among the three species.

My third objective was to identify stages of cellular degradation during the formation of root aerenchyma in *S. lancifolia*. I hypothesized that lysis occurs in a patterned and predictable manner which corresponds to changes previously described for other types of cells undergoing a programmed cell death. Cell structure was examined with TEM (transmission electron microscopy), agarose gels and the TUNEL assay were used to detect DNA fragmentation, and the arrangement of cortical microtubules at different stages of development was documented. In micrographs from the TEM, the primary changes in nuclear structure (including chromatin condensation, membrane disintegration, and apparent nuclear fragmentation) were followed by organelle disintegration and cellular membrane breaks prior to collapse and lysis of the cell. A patterned breakdown of nuclear DNA was not detected by the agarose gels or TUNEL assay. The cellular structure of the final stages of cell lysis in *Z. mays* and *O. sativa*

appeared to differ slightly from each other as well as from *S. lancifolia* but were more similar than expected. Cortical microtubule orientations indicated that diaphragm cells of the cortex had parallel (relative to the root axis) orientations as compared to the diagonal orientations of mature cortex cells. Parallel orientation of microtubules indicates that these cells may expand radially and contribute to the separation of the files of cortical cells that occurs prior to cell lysis and aerenchyma formation.

CHAPTER 2

AERENCHYMA DEVELOPS BY CELL LYSIS IN ROOTS AND CELL SEPARATION IN LEAF PETIOLES IN *SAGITTARIA LANCIFOLIA* (ALISMATACEAE)[1]

Hypoxia in flooded soils inhibits aerobic respiration and produces toxic, reduced compounds in the soil (Crawford, 1982). Aerenchyma tissue, an anatomical adaptation to hypoxia, is characterized by continuous gas spaces in shoots and roots and is a distinctive feature of most wetland species (Esau, 1977). Aerenchyma reduces flooding stress by allowing an internal pathway for oxygen to the root zone to aid in respiration and oxidation of toxic compounds. Despite the importance of aerenchyma to the survival of wetland species, little is known about the processes that lead to its formation in these plants. Only the development of root aerenchyma in the food crops *Zea mays* (Campbell and Drew, 1983) and *Oryza sativa* (Webb and Jackson, 1986) and the formation of diaphragms in the shoots of *Scirpus validus*, and leaves of *Sparganium eurycarpum* and *Typha latifolia* (Kaul, 1971, 1973, 1974) have been investigated.

The formation of aerenchyma gas spaces is thought to occur by either lysigeny or schizogeny (Smirnoff and Crawford, 1983). Lysigenous spaces form via cell lysis and are identified by the presence of cell wall remnants in the gas spaces of mature tissue, while schizogenous spaces form by cells separating during tissue development, producing gas spaces with no cell remnants (Esau, 1977; Smirnoff and Crawford, 1983). Description of gas spaces as "lysigenous" or "schizogenous," however, is usually based on examination of mature tissue, rather than by examination of different stages of tissue

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formation. Certain species, such as corn, normally lack gas spaces but will produce lysigenous gas spaces in roots when grown under stress conditions (principally waterlogging) (Drew, Jackson, and Giffard, 1979; Konings, 1982). Many wetland species, on the other hand, appear to form both root and shoot aerenchyma constitutively (Jackson, 1989). Root gas spaces can be either lysigenous or schizogenous in wetland species (Smirnoff and Crawford, 1983; Justin and Armstrong, 1987). Shoot gas spaces in wetland species usually appear schizogenous (Jackson, 1989), but developmental studies of certain species have found lysigenous formation (Kaul, 1973; Kaul, 1974).

Sagittaria lancifolia is a perennial herb that is a common wetland species of the Northern coast of the Gulf of Mexico, growing in marshes that range from fresh to intermediate salinity (Chabreck, 1972). *S. lancifolia* can be a dominant species in some oligohaline marshes. For example, along the Pearl River in Louisiana, *S. lancifolia* may account for 40-80 % of the plant biomass during the summer (L. Gough, Louisiana State University, personal communication). Reproduction is primarily vegetative, with seedlings rarely occurring in undisturbed, mature communities (A. Baldwin, Louisiana State University, personal communication). Margaret Stant (1964) surveyed the anatomy of several species from the Alismataceae and reported schizogenous aerenchyma in leaves, rhizomes, and roots of *S. lancifolia*. She estimated that the gas space volume encompassed up to 80 % of the total volume in plants of this species.

The objective of this study was to determine the pattern of development of root and shoot aerenchyma gas spaces in a species adapted to flooded soils. We specifically wanted to identify the relative roles of lysigeny and schizogeny in the formation of gas

spaces in the roots and shoots of *S. lancifolia*. We found development of gas spaces in the roots was by lysigeny and development of gas spaces in the shoots was by schizogeny.

MATERIALS AND METHODS

Seedlings of *Sagittaria lancifolia* were purchased (Horticultural Systems, Parrish, FL) and grown in aerated, one-quarter strength Hoagland's solution (Epstein, 1972) in a growth chamber (Environmental Growth Chambers, Chagrin Falls, OH) for 1 month. The photoperiod was 12 h, photosynthetically active radiation at the base of the petioles was 115 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and day and night temperatures were 32 ° and 25 °C, respectively.

The locations of aerenchyma gas spaces in various parts of *S. lancifolia* were determined from hand sections made with razor blades. Plastic-embedded samples were made with tissue sections (1 mm thick) fixed overnight under vacuum in 2 % glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.2 (Tucker, Baird, and Sexton, 1991). Tissue was then rinsed with 50 mM sodium cacodylate buffer, dehydrated in an ethanol series to 100% ethanol and embedded in LR White Resin, medium grade (Electron Microscopy Sciences, Fort Washington, PA). Roots were sectioned at 5-mm intervals from the tip to 90 mm behind the root tip. The youngest developing petiole was sectioned at 2-mm intervals from the base of the meristem to 32 mm above the meristem. Cross and longitudinal sections (1.2 to 1.8 μm thick) were cut with glass knives and a microtome (Sorvall MT-2 "Porter Blum" Ultra-Microtome, Norwalk, CT), stained with toluidine blue and photographed for observation.

The relative gas space volume was estimated in root and petiole sections using tracings of projected images from photographic slides. The total area and gas space areas from each section were cut out, weighed, and the proportion of gas space mass to total mass determined. Measurements of dimensions in these sections were made on the tracings and calibrated with a stage micrometer.

RESULTS

Plants of *S. lancifolia* produced large rhizomes from which leaves arose in an alternate arrangement (Fig. 1). Newly produced leaves were sheathed by the next older leaf (Fig. 1C). A large percentage of the total volume of gas spaces in the plant occurred in the petiole (Fig. 1B), while a smaller percentage occurred in the blade midrib and roots (Fig. 1A, D). There appeared to be no aerenchyma gas spaces in the laminar portion of the blade (Fig. 1A). Observations of fresh sections indicated gas spaces with cell wall remnants in the root tissue and gas spaces without cell wall remnants in the shoot tissue.

Root aerenchyma development--All the cells in the cortex appeared intact in plastic-embedded cross sections from close to the root tip (0 to 20 mm behind it) (Fig. 2). Surrounding the cortex were generally three layers of small cells that may represent a multi-layered epidermis. To the inside of these layers was the first layer of cortical cells, which were slightly larger than the epidermal cells. The next several layers were the largest cells of the cortex and appeared to be arranged in radial files extending inward toward the stele. The radial files were packed side by side with no large spaces between cells (Fig. 2).

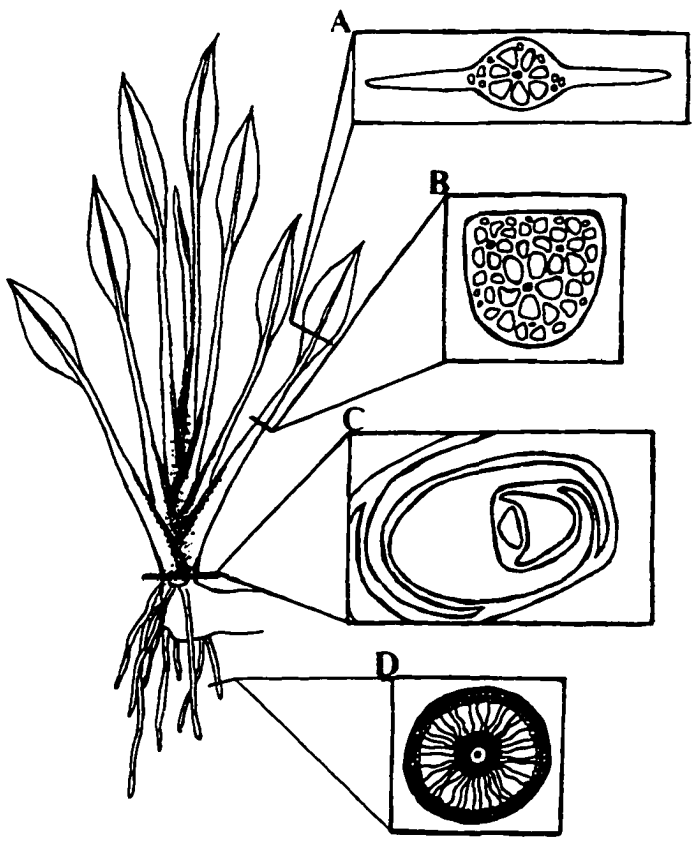
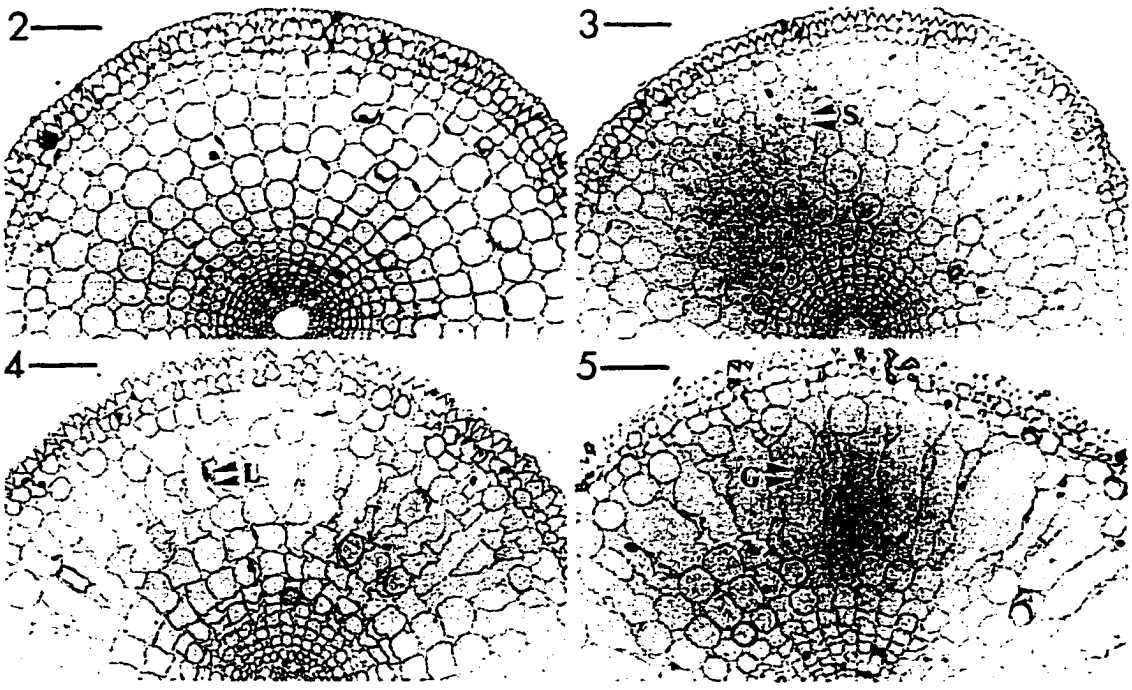


Figure 1. Typical morphology of a *S. lancifolia* plant and cross sections of different plant parts. (A) Cross section of leaf blade. (B) Cross section of petiole. (C) Cross section of shoot meristem. (D) Cross section of mature root.

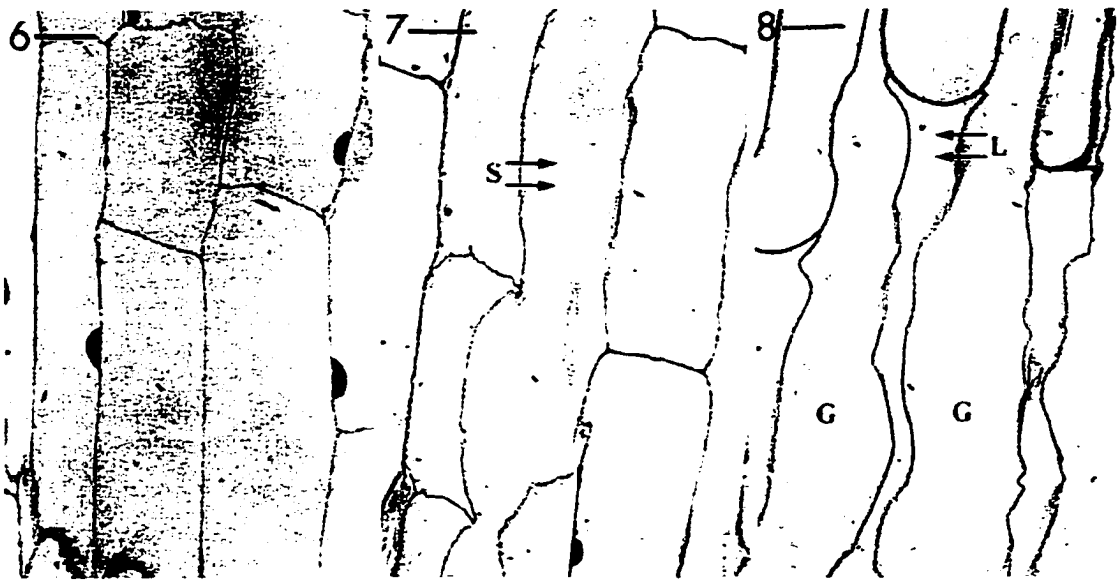


Figures 2-5. Cross sections of roots of *S. lancifolia* at different distances behind the root tip. 2. At 10 mm. 3. At 40 mm. 4. At 60 mm. 5. At 85 mm. Bars = 100 μm. L, lysis; G, gas space; S, space between files.

In cross sections from 20 to 40 mm behind the tip, the radial files of cells appeared slightly separated in some areas of the outer cortex (Fig. 3). The spaces between radial files of cells became larger as the distance from the root tip increased. Cell lysis was observed in a few random locations in sections 20 to 40 mm behind the root tip, again in the outer cortex (Fig. 3). In cross sections located between 40 mm and the point where the root attached to the rhizome, certain cells in the cortex region looked deflated or shriveled (Fig. 4).

The volume occupied by gas spaces increased as distance behind the root tip increased and did not reach a maximum until the point where the root attached to the rhizome, about 90 mm behind the root tip (Fig. 5). Generally, the 2 to 4 largest cells in radial files closest to the epidermis appeared to have lysed, leaving intact the smaller three to four layers of cells to the outside of the cortex that were not in radial rows. Where cortical cells lysed, a strip of cell wall material remained, linking intact inner and outer cortical cells through the gas spaces (Fig. 5).

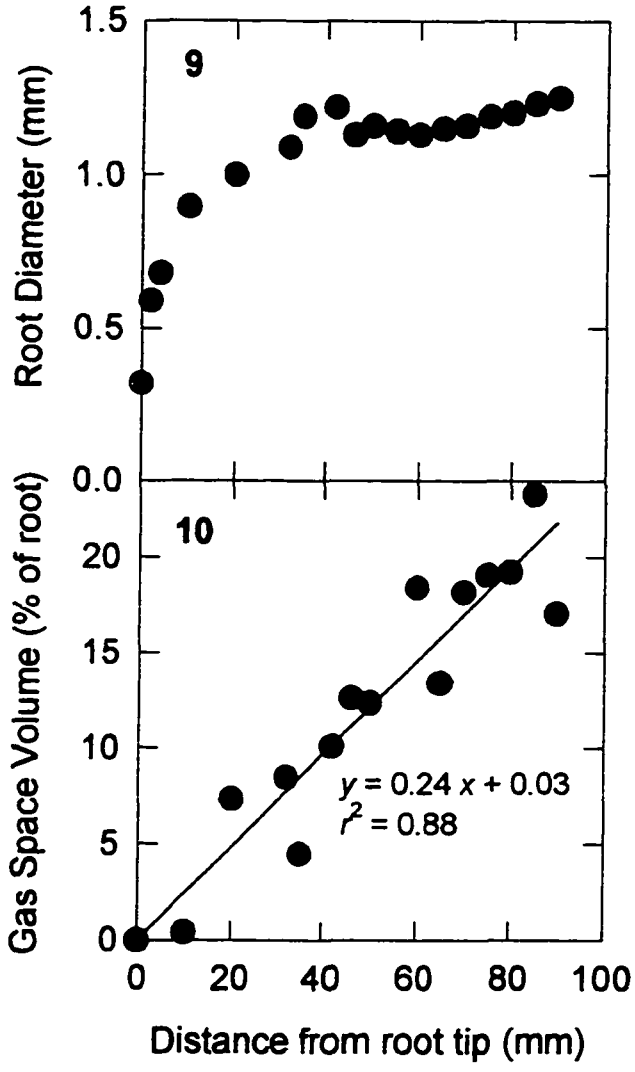
Observations of longitudinal sections confirmed findings from cross sections. Between the root tip and 20 mm behind the tip, there were few intercellular spaces and cortical cells were tightly packed (Fig. 6). In sections from > 20 mm behind the root tip, cortical cells began to separate and some cells appeared deflated, indicating cell lysis (Fig. 7). The maximum volume of gas space was found in longitudinal sections closest to the point where the root emerged from the rhizome (Fig. 8). Cortical cells appeared to lyse along most of the root. The number of cells appearing to lyse and the degree of disintegration increased from the root tip to the rhizome junction.



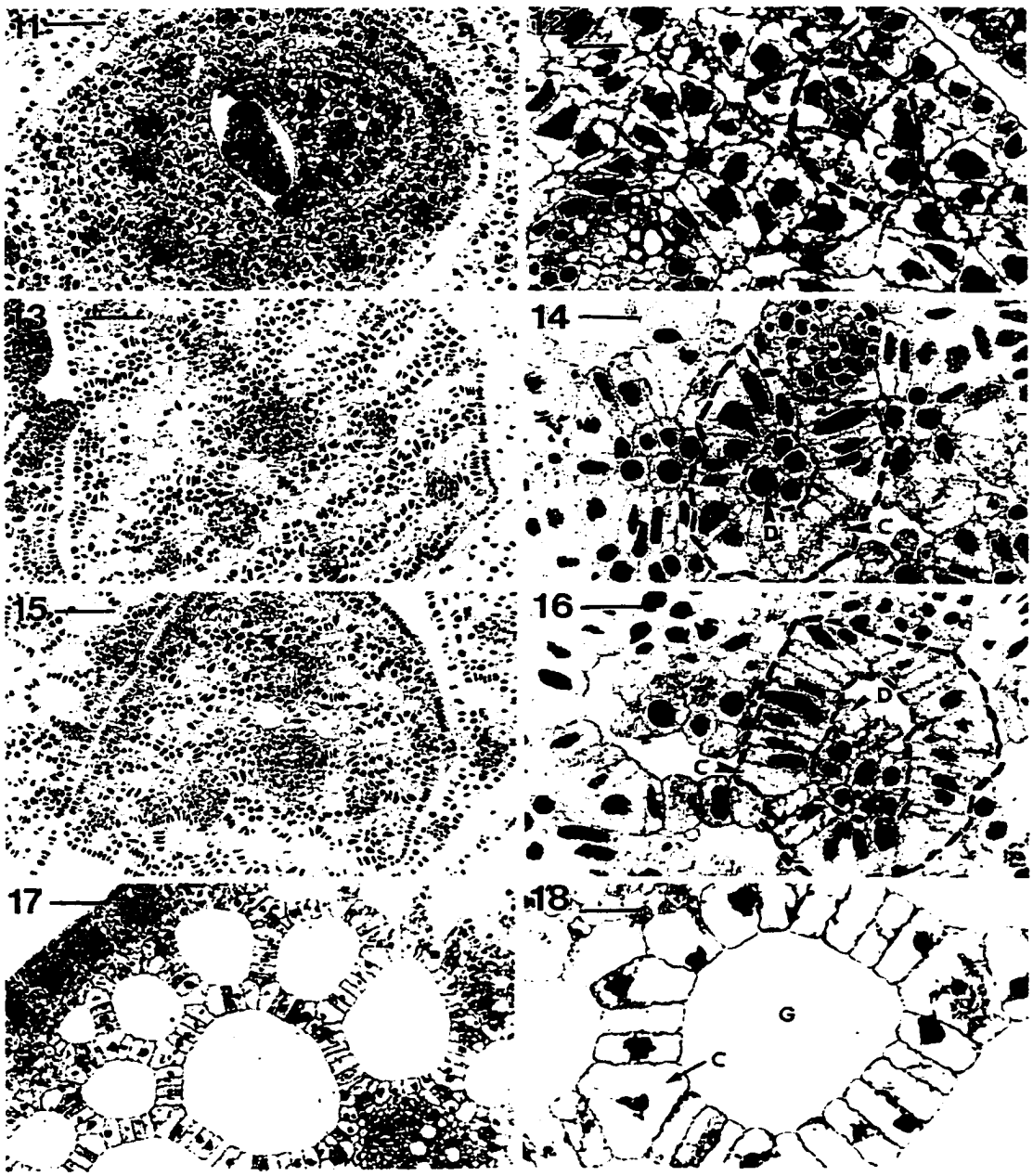
Figures 6-8. Longitudinal sections of roots of *S. lancifolia* at different distances behind the root tip. 6. At 20 mm. 7. At 42 mm. 8. At 80 mm. Bars = 25 μm. L, Lysis; G, gas space; S, space between files.

The relationships between the distance from the root tip and both root diameter and gas space volume were measured in root cross sections. Root diameter increased from the root tip to about 40 mm behind the tip (Fig. 9). The number of cells per file and the number of files in root sections also increased (data not shown), consistent with cell division being an important component of radial growth. Beyond 40 mm there was little increase in root diameter (Fig. 9) or number of cells in a file and number of cell files (data not shown). The root volume occupied by aerenchyma gas spaces, however, continued to increase almost linearly (gas space volume [%] = 0.24 x distance from the root tip [mm] + 0.03; $r^2 = 0.88$) to a maximum volume of about 25 % at 90 mm (Fig. 10).

Petiole aerenchyma development--Cortical cells were packed tightly with only occasional, small spaces between cells in cross sections from 2 mm above the meristem (Figs. 11, 12). There were groups of wedge-shaped cells that fit together to form cylinders (Fig. 12). Many of these cylinders appeared to share cells with each other in the developing cortex and thus appeared "interlocked." From 2 to 4 mm above the meristem the diameter of cylinders increased and the cells making up the cylinders became more rectangular in shape (Figs. 13, 14). The number of cells making up each cylinder increased from an average of seven cells per cylinder at 2 mm to 16 cells per cylinder at 4 mm and the average inner diameter of each cylinder increased from 12 mm to 40 mm at 2 mm and 4 mm, respectively. There were groups of small, round cells (diaphragm cells) in the middle of each cylinder at 4 mm (Fig. 14).



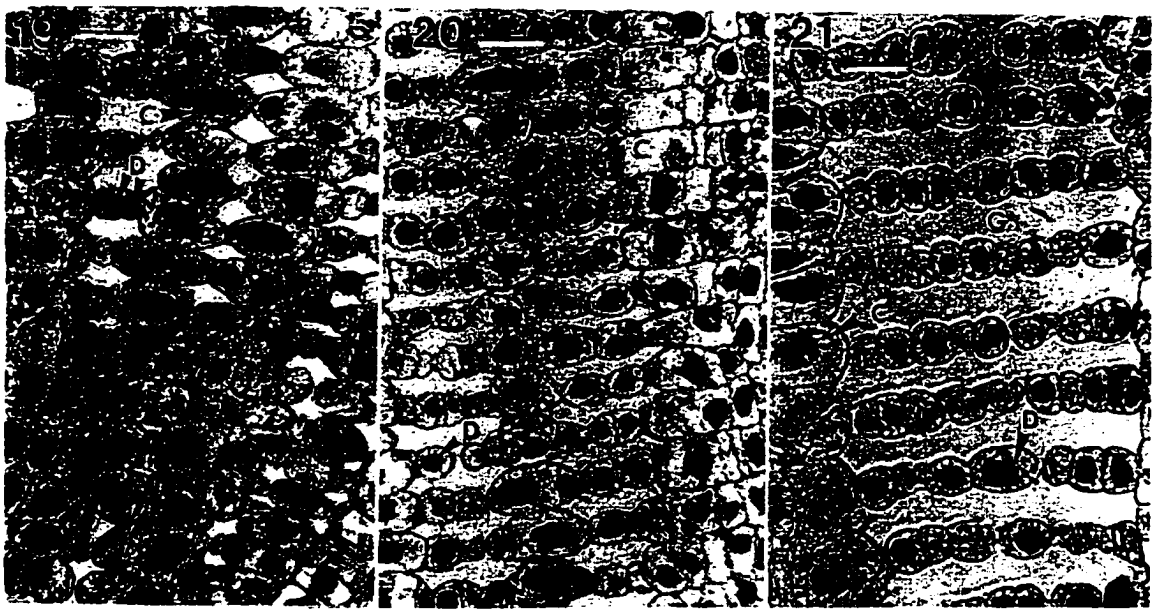
Figures 9-10. Root diameter measurements (9) and calculated volumes of root gas space (percent of total root volume) (10) at different distances behind the root tip in *S. lancifolia.*



Figures 11-18. Cross sections of petioles of *S. lancifolia* at different distances above the shoot meristem at two magnifications. 11. At 2 mm above the meristem. 12. Enlargement of Figure 11. 13. At 4 mm above the meristem. 14. Enlargement of Figure 13. 15. At 6 mm above the meristem. 16. Enlargement of Figure 15. 17. At 12 mm above the meristem. 18. Enlargement of Figure 17. Bars = 100 μm in Figures 11, 13, 15, 17. Bars = 25 μm in Figures 12, 14, 16, 18. C, cell of a cylinder; D, diaphragm cell; G, gas space. A cylinder outlined by a dashed line on Figures 12, 14, and 16.

In cross sections 6 mm above the meristem (Figs. 15, 16), the average number of cells making up a cylinder (23) and the average inner diameter of a cylinder (66 mm) were greater than in the 4-mm sections. Gaps and cells that appeared lysed were observed in the diaphragm layer in the middle of some cylinders when the plane of the section was not the same as that occupied by the diaphragm. By 12 mm above the meristem, there were few, if any, diaphragm cells in the middle of the cylinders and cylinders were clearly interlocked (Figs. 17, 18). In a mature cross section of petiole tissue there could be 50 to 60 cylinders, each cylinder being made up of an average of 35 cells and having an average inner diameter of 236 mm.

Longitudinal sections of petioles presented a clearer view of how gas spaces developed. Close to the shoot meristem, cortical cell initials appeared in files parallel to the petiole axis and as the distance above the meristem increased these cells appeared to develop into two cortical cell types (Fig. 19). In longitudinal sections 0.5 mm above the meristem (Fig. 19), there were files of cells parallel to the petiole axis that corresponded to the cells making up the cylinders in cross sections. Between some of these cells making up the cylinders there were one or two round cells oriented perpendicular to the petiole axis that were the initial diaphragm cells. At about 2 mm above the meristem (Fig. 20), there were two or three diaphragm cells between each cell that made up the cylinders. Each diaphragm layer was very close to the next layer above it, but they never appeared to touch, producing slight schizogenous gaps. By 4 mm above the meristem, the gas spaces between diaphragm layers were more prominent and each diaphragm contained seven to ten cells (Fig. 21). At 4 mm, the cells making up the



Figures 19-21. Longitudinal sections of petioles of *S. lancifolia* at different distances above the shoot meristem. 19. At 0.5 mm above the meristem. 20. At 2 mm above the meristem. 21. At 4 mm above the meristem. Bars = 25 μm. C, cell of a cylinder; D, diaphragm cell; G, gas space.

cylinders were larger than in earlier stages and some of these cells had divided and did not have a diaphragm connected to them. This produced larger gaps between diaphragms than those found closer to the meristem.

DISCUSSION

Root gas spaces were formed by lysigeny and petiole gas spaces were formed by schizogeny. The aerenchyma of the root and petiole may have different mechanisms of gas space formation which could have implications for different types of stress adaptation in each tissue.

In the roots, there was only one cell type in the cortex and aerenchyma development appeared to be by lysis. Near the root meristem there was a central vascular cylinder and parenchyma cells arranged in radial files extending from the vascular cylinder to the outer cortex. In older root tissue there was almost complete lysis of the cortical cells that were in radial files near the outer cortex. Root gas space formation appeared to begin with the appearance of slight schizogenous gaps between the radial files. Whether this was a necessary precursor to the lysis that followed is unknown. Remnants of cell walls generally formed radial "strands" that connected the living outer and inner cortical cells.

The locations of cells undergoing lysis appeared precise, indicating a targeting mechanism for lytic enzyme(s). Studies (not of wetland species) suggest that enzymatic digestion of cell walls by specific cellulases produce cell lysis (Kawase, 1979). In this study of *S. lancifolia* roots, the cells undergoing lysis appeared to be larger in diameter than the cortex cells that remained intact, so cell size may be related to cell lysis. Wong

and Osborne (1978) also reported cells that lysed in the fruit abscission zone of *Ecballium elaterium* were larger than cells that remained intact.

Mechanisms controlling cell death to form gas space tissue are not well understood. Studies on corn and sunflower (*Helianthus annuus*) have led to the hypothesis that hypoxia produces an increase in ethylene concentration (Drew, Jackson, and Giffard, 1979; Konings, 1982), which leads to cell death and the lysis of cell walls and protoplasts. Part of the degradation process involves the action of cellulases (Kawase, 1979; He, Drew, and Morgan, 1994). In corn, suboptimal concentrations of nitrogen and phosphate also induce aerenchyma by apparently increasing sensitivity to ethylene (He, Morgan, and Drew, 1992). Development of root aerenchyma has been studied in the agronomically important species *Zea mays*, corn (Campbell and Drew, 1983) and *Oryza sativa*, rice (Webb and Jackson, 1986). Both species form lysigenous root aerenchyma that appears similar in structure, but there are some significant differences in cell appearance prior to lysis and in the effect of environmental factors on gas space volume. In lysing cells of corn roots, the tonoplast ruptures and the cell collapses before breakdown of the cell wall (Campbell and Drew, 1983). In rice, however, the cell wall breaks down before loss of tonoplast integrity (Webb and Jackson, 1986). Aerenchyma forms only under hypoxia or high ethylene concentration in corn (Drew, Jackson, and Giffard, 1979). Studies of two different rice cultivars found that oxygen concentration had no effect on the formation and amount of root porosity (Varade, Letey, and Stolzy, 1971; Jackson, Fenning, and Jenkins, 1985). Three other studies (one with four different varieties of rice), however, found changes in the amount of root porosity with the degree of hypoxia (Das and Jat, 1977; Justin and

Armstrong 1991; Kludze, DeLaune and Patrick, 1993). These studies have confirmed that rice always forms gas spaces, but the relative volume of gas spaces can vary with the amount of flooding and the rice variety. These differences in the formation of root aerenchyma in corn and rice may indicate two types of lysigeny regulated by different mechanisms.

Like rice, many wetland species consistently produce root aerenchyma, but gas space volume may vary with environmental conditions. For example, the volume of root aerenchyma produced under different periods of flooding varies in the wetland species *Spartina patens* (Burdick and Mendelssohn, 1987) and *Scirpus americanus* (Seliskar, 1988). Preliminary studies of *S. lancifolia* roots and shoots under varying oxygen concentrations indicate that root gas spaces, but not shoot gas spaces, can fluctuate significantly in their volume (E. E. Schussler and D. J. Longstreth, unpublished data). Future study of the influence of environmental variation on formation of gas spaces in wetland species, especially gas spaces of shoots, is required.

The petioles of *S. lancifolia* have a developmental pattern that is based on cell separation, division, and expansion rather than cell lysis. The development of two types of cortical cells produced gas spaces in leaf petioles as summarized in Fig. 22. The pattern of gas spaces is clearly established at a very young developmental age by the formation of two different cell types. Cells making up the cylinders, as well as cells of the diaphragms, undergo division both parallel and perpendicular to the petiole axis to increase the volume of gas spaces during development. Close to the meristem,

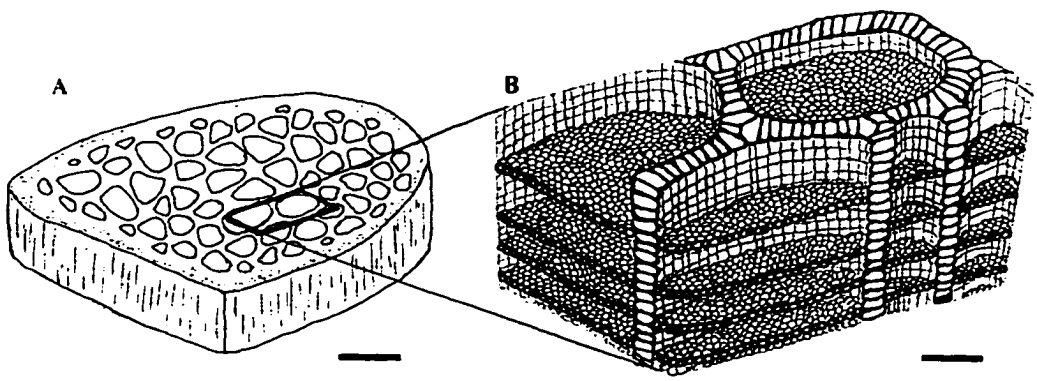


Figure 22. Diagram of petiole gas space arrangement in *S. lancifolia*. (A) Representation of petiole cross section with gas spaces. Bar = 1 mm. (B) Expanded section of petiole showing gas spaces and diaphragms. Bar = 200 μm.

diaphragms within a cylinder are very close to one another, but with petiole maturation the distance between diaphragms increases to produce large gas spaces.

In contrast to the formation of gas spaces by schizogeny in the petiole of *S. lancifolia*, Kaul (1971, 1973, 1974) reported gas spaces formed by lysigeny in the leaves of *Sparganium eurycarpum* and *Typha latifolia*, and shoots of *Scirpus validus*. In these species, certain derivatives of the rib meristem differentiate into diaphragm cells and then the undifferentiated derivatives lyse, leaving gas spaces partitioned by the diaphragms. As the stems or leaves elongate, there is separation of diaphragms. The mechanism producing cell breakdown in *S. eurycarpum*, *T. latifolia*, and *S. validus* has not been studied.

Further studies are needed to determine if production of gas spaces by both lysigeny and schizogeny is common in other wetland species. There is clearly diversity in the way gas spaces are formed and evaluation of this diversity should improve our understanding of plant adaptation to flooded conditions. Understanding the biochemical and molecular basis of lysigeny and schizogeny may also provide important insights into the control of cell development, senescence, and death.

CHAPTER 3

ENVIRONMENTAL FACTORS AFFECTING AERENCHYMA DEVELOPMENT

Aerenchyma (or gas-space) tissue, a plant adaptation for growth in waterlogged conditions, provides an internal pathway for O_2 transport to roots. Species adapted to waterlogged environments usually produce aerenchyma under all conditions and this can be described as a "constitutive" production of aerenchyma (Jackson, 1989; Drew, 1997). However, the percentage of the total volume occupied by gas spaces (subsequently referred to as "relative volume" of aerenchyma) may change with aeration status of the soil in certain species (Smirnoff and Crawford, 1983; Burdick and Mendelssohn, 1987). For example, *Oryza sativa* (rice) is adapted to waterlogged conditions and has constitutive root and shoot aerenchyma, but depending on the variety, the relative volume of root aerenchyma may (Das and Jat, 1977; Justin and Armstrong, 1991; Kludze, DeLaune, and Patrick, 1993) or may not (Varade, Letey, and Stolzy, 1971; Jackson, Fenning, and Jenkins, 1985) increase as oxygen in the soil is reduced.

Helianthus annuus (sunflower), *Lycopersicon esculenteum* (tomato), and *Zea mays* (corn) have root aerenchyma when grown in waterlogged conditions, but not in well-aerated conditions (Drew, Jackson, and Giffard, 1979; Kawase, 1979; Kawase and Whitmoyer, 1980). This can be described as "inducible" production of aerenchyma.

In *Z. mays*, root aerenchyma is formed by cell lysis which may be regulated by ethylene concentration (He, Drew, and Morgan, 1994; He, Morgan, and Drew, 1996). Under hypoxic conditions, ethylene concentration increases in the roots of *Z. mays*

(Atwell, Drew, and Jackson, 1988). This increase in concentration appears to trigger an increase in cellulase activity, which is postulated to cause cell lysis and aerenchyma formation (He, Morgan, and Drew, 1992; He, Drew, and Morgan, 1994). In *Z. mays*, root cortex cells can lyse at ethylene concentrations that do not affect surrounding cells (He, Morgan, and Drew 1992, Baluska et al., 1993). This suggests that cell lysis could be regulated by sensitivity to ethylene rather than concentration of ethylene. However, aerenchyma formation in at least one variety of *O. sativa* is not affected by changes in ethylene concentration, suggesting a pathway for cell lysis that does not depend on regulation by ethylene (Jackson, Fenning, and Jenkins, 1985).

Although changes in soil aeration appear to affect aerenchyma formation differently in species adapted to waterlogging and species not adapted to waterlogging, direct comparisons among species are rare. The objective of this chapter was to determine how growth at different O_2 concentrations affects aerenchyma formation in *Sagittaria lancifolia*, and how this response compares to that in *Z. mays* and *O. sativa*. Root aerenchyma in *Sagittaria lancifolia* is formed by cell lysis and petiole aerenchyma by cell separation (Chapter 2). Observations of *S. lancifolia* plants growing under a variety of flooding regimes in the field, indicated formation of root and petiole aerenchyma is produced in a constitutive fashion in this species. Results of this study suggested that the process of cell lysis to form aerenchyma can be significantly different among species.

MATERIALS AND METHODS

Plant Material

Sagittaria lancifolia plants (Horticultural Systems, Parrish, FL) were grown in one-quarter strength Hoagland's solution (Epstein, 1972) with constant aeration for at least two weeks prior to the experiment. Seeds of *Z. mays* and *O. sativa* (cv. Labell) were germinated in moist vermiculite in a growth chamber (Environmental Growth Chambers, Chagrin Falls, OH). Two-week old seedlings of *Z. mays* and *O. sativa* were transferred to modified Hoagland's solutions (Yoshida et al., 1976; Drew, Jackson, and Giffard, 1979) with constant aeration for at least two weeks prior to the experiments. All experiments were conducted in an environmental growth chamber with a photoperiod of 12 h, photosynthetically active radiation at the base of the petiole of 115 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and day/night temperatures of 32 and 25 °C, respectively.

O_2 and Ethylene Treatments

Plants were grown in plastic containers (3.5 L) and air or N_2 gas was bubbled into the hydroponic solution to produce different O_2 concentrations in the root zone. Sixteen *S. lancifolia* plants were grown in solutions with normal or low O_2 and ethylene concentrations in four treatments. For the normal O_2 treatment, air was bubbled continuously into the hydroponic solutions, yielding an O_2 concentration between 9-10 ppm. For the low O_2 treatment, N_2 gas was bubbled into the hydroponic solutions approximately every twelve hours for 30 min producing an O_2 concentration between 3-5 ppm. For the normal ethylene treatment, the hydroponic solution was unaltered. Silver nitrate, which inhibits ethylene binding, and aminoethoxyvinylglycine (AVG),

which inhibits ethylene formation, were added to the nutrient solution in 0.5 μM and 0.1 μM final concentrations, respectively, to produce the low ethylene treatment (He, Drew, and Morgan, 1994). After four weeks of growth, the roots and petioles of the plants were sampled for relative volume of aerenchyma and cellulase activity. Statistical analyses were conducted using a two-by-two factorial ANOVA. The experiment of growing plants in normal and low O_2 treatments was repeated.

For comparison with *S. lancifolia*, *O. sativa* and *Z. mays* plants were grown in normal and low O_2 treatments (as described above) in another experiment. The effect of ethylene concentration was not tested for either *Z. mays* or *O. sativa*. After one week of treatment, the relative volume of root aerenchyma and the cellulase activity in root tissue were determined for each species. The experiments were repeated. For each species, a t-test was used to test for significant differences between means of relative aerenchyma volume and cellulase activity for the two O_2 treatments.

Relative Volume Measurements

In each species, tissue samples were collected from mature tissue of young, white roots where previous measurements showed the relative volume of aerenchyma was greatest. Petiole tissue in *S. lancifolia* was sampled directly above the sheath. Petiole tissue was hand sectioned and photographed for calculation of the relative volume of aerenchyma. Root pieces of 2-3 mm were fixed overnight, under vacuum, in 2 % glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.2. The tissue was rinsed in the buffer, dehydrated in an ethanol series, and embedded in LR White Resin, medium grade (Electron Microscopy Sciences, Fort Washington, PA). Cross sections

(1.2-1.8 μm) were cut with glass knives on a microtome (Sorvall MT-2 "Porter Blum" Ultra Microtome, Norwalk, CT) and stained with toluidine blue. Cross-sections were then observed with a light microscope and photographed. Slides of root and petiole cross sections were projected and traced, and the area occupied by gas spaces relative to the total area of the tissue section was used to determine relative volume of aerenchyma. Measurements of dimensions in these sections were made on the tracings and calibrated with a stage micrometer.

Cellulase Assay

Cellulase activity was assayed in root and petiole tissue by a viscometric assay (Durbin and Lewis, 1988) as modified by del Campillo and Lewis (1992), and He, Drew, and Morgan (1994). Briefly, tissue samples of approximately 0.5 g were ground on ice with buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl). The extract was centrifuged at 3000 g for 10 min. Two mL of the supernatant were combined with 4 mL of carboxymethylcellulose (CMC) solution (1.3 % CMC in 0.02 M Tris-HCl, pH 7.5), and the initial viscosity was determined with a viscometer. After viscosity was again measured 60 min later, the change in viscosity was determined and used to calculate the relative activity of cellulase per gram of tissue. These results were standardized to known concentrations of cellulase (Cellulase (EC 3.2.1.4) from *Trichoderma viride*, Sigma Chemical Co., St. Louis, MO).

RESULTS

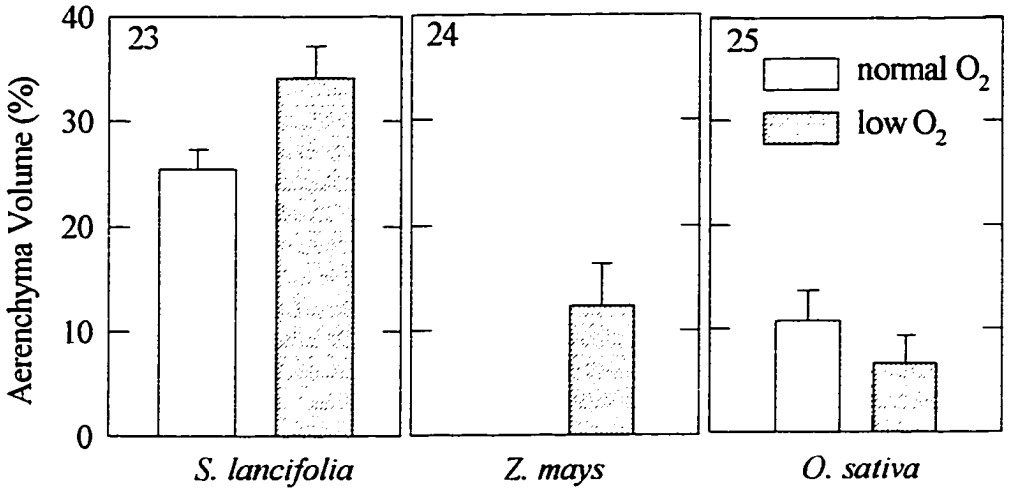
Relative Volume of Root Aerenchyma and O_2 Treatment

In *S. lancifolia*, aerenchyma was produced in roots grown in both normal and low O_2. The mean relative volume of aerenchyma tissue was significantly greater (p = 0.03) in roots from the low O_2 treatment (Fig. 23). The mean area of cross-sections was 1.10 $mm^2 \pm 0.08$ (SE) from roots grown in normal O_2, and 1.69 $mm^2 \pm 0.12$ (SE) from roots grown in low O_2.

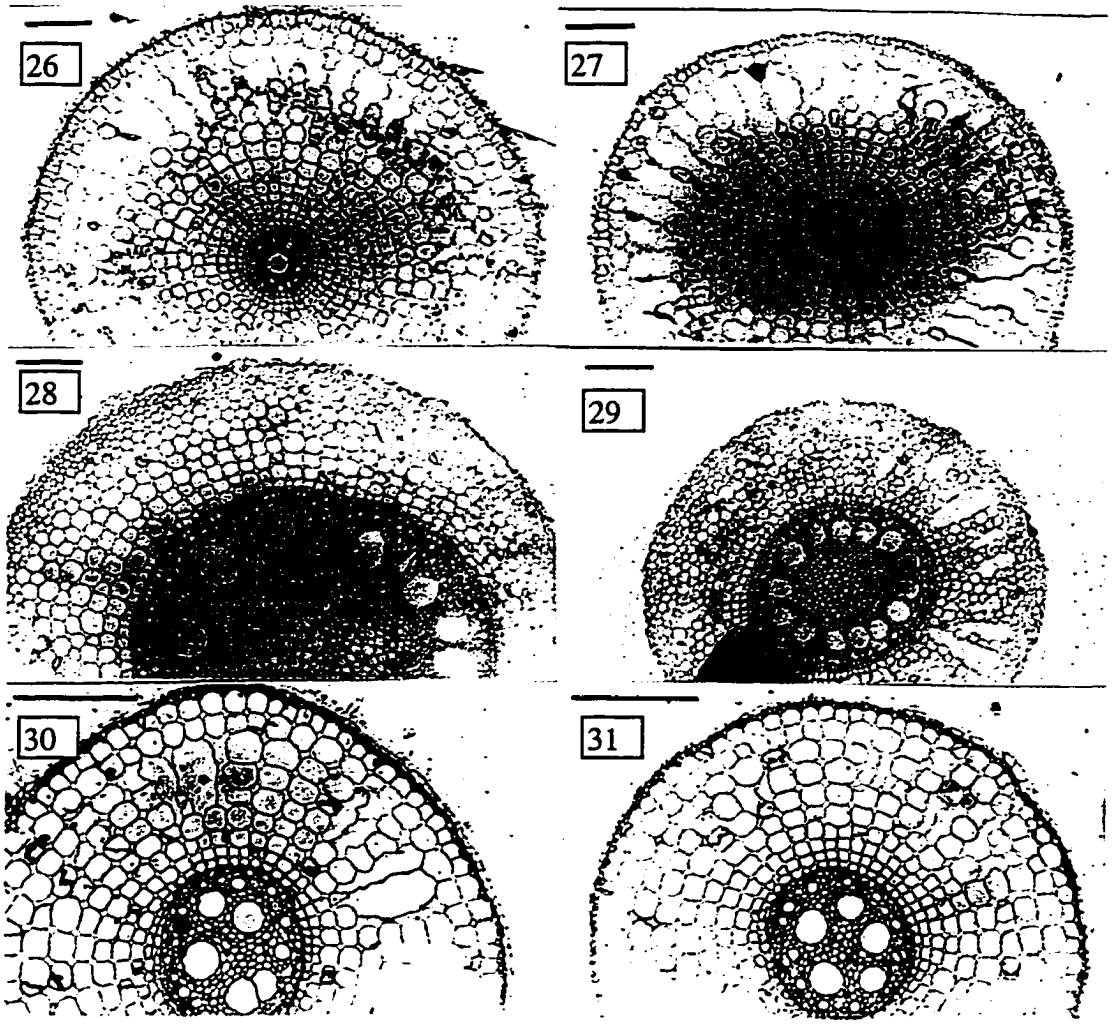
In *Z. mays*, aerenchyma was formed in roots grown in low O_2, but not in roots grown in normal O_2 (Fig. 24). The mean area of cross-sections was 0.43 $mm^2 \pm 0.06$ (SE) from roots grown in normal O_2, and 0.55 $mm^2 \pm 0.06$ (SE) from roots grown in low O_2.

In *O. sativa* cv. Labell, aerenchyma was produced in roots grown in both normal and low O_2. The relative volume of aerenchyma produced in both treatments was highly variable, and the difference between these means was not significant (Fig. 25). The mean area of cross-sections was 0.36 $mm^2 \pm 0.04$ (SE) from roots grown in normal O_2, and 0.42 $mm^2 \pm 0.03$ (SE) from roots grown in low O_2.

All cortex cells in *S. lancifolia* and *O. sativa* were aligned in radial files (rows) (Figs. 26, 27, 30, 31), while cortex cells in *Z. mays* were occasionally arranged in radial files (Figs. 28, 29). Cell lysis in *S. lancifolia* and *O. sativa* produced gas spaces in the outer cortex that were delineated by radial rows of collapsed cells. Gas spaces in *Z. mays* were also concentrated in the outer cortex, but collapsed cells did not remain in radial rows.



Figures 23-25. Relative aerenchyma volumes (gas space percentage of cross-sectional area) in *S. lancifolia* (23), *Z. mays* (24), and *O. sativa* (25) roots grown at normal O_2 (continuously bubbled with air) and low O_2 (bubbled with nitrogen twice daily). Means represent eight replicates ± the standard error. Means were significant for *S. lancifolia* ($p = 0.03$) and *Z. mays* ($p < 0.01$), but not for *O. sativa*.



Figures 26-31. Representative cross sections of root tissue after growth in different O_2 concentrations. 26. *S. lancifolia* grown in normal O_2. 27. *S. lancifolia* grown in low O_2. 28. *Z. mays* grown in normal O_2. 29. *Z. mays* grown in low O_2. 30. *O. sativa* grown in normal O_2. 31. *O. sativa* grown in low O_2. Bars = 200 μm.

Root Cellulase Activity and O_2 Treatment

Differences between the mean cellulase activity from root tissue grown in normal and low O_2 were not significantly different for any of the three species (Table 1). Mean root cellulase activities from *Z. mays* and *O. sativa* were variable, but higher than the mean cellulase activities in *S. lancifolia* roots (Table 1).

Petiole Aerenchyma in *S. lancifolia*

The mean relative volume of aerenchyma in petioles of *S. lancifolia* was not affected by changing O_2 concentration in the root zone. The mean relative volume of aerenchyma was 41.2 % ± 2.3 (SE) in petioles from plants grown in normal O_2 and 41.2 % ± 1.4 (SE) in petioles from plants grown in low O_2. Petioles from plants grown in normal O_2 had a mean cellulase activity of 0.36 ± 0.03 (SE) units per gram of tissue, while petioles from plants grown in low O_2 had a mean activity of 0.30 ± 0.03 (SE) units per gram of tissue. Mean cellulase activity in petioles was about 40 % lower than mean cellulase activity in roots (Table 1). Neither mean relative volume of aerenchyma nor mean cellulase activity in roots and petioles of *S. lancifolia* were significantly reduced in hydroponic solutions containing ethylene inhibitors (data not shown).

DISCUSSION

Aerenchyma was produced in roots of *S. lancifolia* and *O. sativa* under all treatment conditions. In contrast, aerenchyma was only produced in roots of *Z. mays* grown in the low O_2 concentration. Aerenchyma is formed by cell lysis in the roots of all three species, so there are apparent differences in how the process of cell lysis is

Table 1: Cellulase activity (enzyme units · g^{-1} fresh weight) in roots of *S. lancifolia*, *Z. mays*, and *O. sativa* from normal and low O_2 treatments. The data presented are means of eight replicates ± the standard error. There were no significant differences between means from roots grown in normal and low O_2 for any species.

	normal O_2	low O_2
S. lancifolia	0.66 ± 0.12	0.43 ± 0.08
Z. mays	2.72 ± 1.61	4.28 ± 1.89
O. sativa	5.53 ± 1.48	5.83 ± 2.58

regulated. There are also different responses among species when roots are grown at different ethylene concentrations. The formation of root aerenchyma in *Z. mays* is induced by ethylene (Drew, Jackson, and Giffard, 1979; He, Morgan, and Drew, 1992), while formation of root aerenchyma in *O. sativa* (Jackson, Fenning, and Jenkins, 1985) and *S. lancifolia* (in this study) is not affected by ethylene.

One explanation for different patterns of root aerenchyma development among species is that the cellular events producing cell lysis are the same, but the initiation of the sequence of events can be regulated differently. Ethylene is thought to trigger formation of root aerenchyma in *Z. mays* (He, Morgan, and Drew, 1996), and may also trigger root aerenchyma formation in species that appear insensitive to ethylene. Multiple genes coding for putative ethylene receptors have been identified in *Arabidopsis* (Chang et al., 1993) and the gene products appear to be dimerized when they are active (Kieber, 1997). Differential synthesis of receptors, and dimerization of different combinations of gene products, may cause differential ethylene "sensitivity" among cells (Kieber, 1997). In *Z. mays*, differences in cell response to ethylene have been documented (Baluska et al., 1993). If cortex cells in *S. lancifolia* and *O. sativa* are very sensitive to ethylene, cell lysis could be triggered by such small concentrations of ethylene that they could appear insensitive to ethylene concentration relative to cortex cells from *Z. mays*. Alternatively, differences among the species could be a result of differences in regulation of the actual pathway, rather than regulation of the initial signal. Constitutive expression of the ethylene triple response due to a defect in the ethylene signal transduction pathway has been reported in mutants of *Arabidopsis* (Kieber et al., 1993). A defective negative regulator of the pathway, downstream from

the receptor, does not turn off the pathway in these mutants when ethylene is absent, thus producing a constitutive phenotype. Therefore the same pathway may operate in all three species, but in *S. lancifolia* and *O. sativa* aerenchyma is always produced because these species lack a negative regulator as in the case of *Arabidopsis* mutants for ethylene triple response.

A second explanation for the differences in root aerenchyma development among *S. lancifolia*, *O. sativa* and *Z. mays* would be the existence of completely different cellular signaling pathways leading to cell lysis. There is currently no direct evidence to support this hypothesis, but results from studies of programmed cell death (PCD) may provide indirect support. Discovery of multiple genes controlling the PCD that is part of the hypersensitive response to pathogen attack has led to speculation that PCD occurs by more than one mechanism (Greenberg, 1996). Clarke (1990) has documented at least three morphologically distinct sequences of PCD in animal cells. In their review of PCD in plants, Jones and Dangl (1996) cite the induction of cell death by both intrinsic and extrinsic signals and variations in cell morphology during cell death as evidence for the existence of more than one pathway of PCD.

In *Z. mays*, aerenchyma formation and cellulase activity are positively correlated with low O_2 concentration (He, Drew, and Morgan, 1994). In the present study, cellulase activities in roots of *Z. mays* grown in low O_2 were not significantly higher than those grown in normal O_2. In *S. lancifolia* and *O. sativa*, mean cellulase activities for each treatment were not significantly different, consistent with constitutive production of root aerenchyma under all treatments. Whether the cellulase activity

found in roots of *S. lancifolia* and *O. sativa* plays a role in producing the root aerenchyma is unknown.

There was constitutive production of root aerenchyma in *S. lancifolia* and *O. sativa*. However, the relative volume of root aerenchyma increased in *S. lancifolia* grown at low O_2 treatment, but O_2 concentration did not affect the relative volume of root aerenchyma in *O. sativa* (also, Jackson, Fenning, and Jenkins, 1985). Flooding also results in an increase in the relative volume of gas spaces in other marsh species, such as *S. patens* (Burdick and Mendelssohn, 1987) and some flood-tolerant British wetland species (Smirnoff and Crawford, 1983). Therefore there appear to be at least two types of constitutive production of root aerenchyma, one shown by species in which the relative volume of aerenchyma increases as O_2 concentration decreases (e.g., *S. lancifolia*) and another shown by species for which the relative volume of aerenchyma remains constant (e.g., *O. sativa*)

Unlike the response of root aerenchyma to O_2 treatment in *S. lancifolia*, cell separation to form petiole aerenchyma in this species was unaffected by changes in the O_2 concentration of the root zone. The large relative volume of the petiole aerenchyma is testimony to the importance of shoot aerenchyma for gas transport to the roots (see Dacey, 1980). The mean relative volume of aerenchyma in the petioles (40 %) was consistently higher than the mean relative volume in the roots (about 30 %). The tall form of *Spartina alterniflora* also has a larger relative volume of aerenchyma in the stem bases (42 %) than in the roots (29 %) (Arenovski and Howes, 1992).

These results demonstrate differences among species in the mechanisms controlling cell lysis and the formation of root aerenchyma. These differences may be a

result of differential regulation of a single pathway leading to cell death, or may represent different pathways leading to cell death.

CHAPTER 4

STRUCTURAL CHANGES IN CELLS LYSING TO FORM ROOT AERENCHYMA

The lysis of cells during the formation of root aerenchyma may be a type of programmed cell death (PCD) (Jones and Dangl, 1996; Drew, 1997). Root aerenchyma in the marsh species *Sagittaria lancifolia* is formed under all conditions in a process that can be termed constitutive cell lysis (Chapter 3). The objective of this research was to compare cell lysis in *S. lancifolia* to the general characteristics reported in examples of programmed cell death.

During PCD, cell structure deviates from that in normal, healthy cells. In animal cells, structural changes observed during cell death have been characterized as apoptotic or non-apoptotic. The morphological characteristics of apoptotic cell death are cell shrinkage, condensation of nuclear chromatin (pycnosis), fragmentation of the nucleus, and outward membrane blebbing (exocytosis of cell contents in membrane-bound "apoptotic" bodies) (Kerr, Wyllie, and Currie, 1972). During apoptosis, DNA degradation by endonucleases can be detected by the TUNEL (TdT-mediated nick end labeling) assay for nicked, 3′ DNA or the formation of a "DNA ladder" on an agarose gel. Animal cell death without apoptotic morphology and detectable DNA fragmentation is called non-apoptotic (Schwartz et al., 1993). Two non-apoptotic morphologies have been identified: autophagic degeneration and non-lysosomal vesiculate degeneration (Clarke, 1990). In autophagic degeneration, numerous autophagic vacuoles destroy the cell contents. In non-lysosomal vesiculate degeneration, swelling of the organelles is followed by the formation of empty spaces in

the cytoplasm and the disintegration of cell contents without the apparent action of lysosomes.

In plants, cell morphology during PCD is mostly non-apoptotic. During tracheary element differentiation in *Zinnia elegans*, cellular organelles degrade after the tonoplast breaks, and degradation of the nucleus is not preceded by nuclear shrinkage and fragmentation (Fukuda, 1997; Groover et al., 1997). Although this morphology is considered non-apoptotic, the TUNEL assay labels nuclei of these cells (Groover et al., 1997). Similarly, cell death during the hypersensitive response to pathogen attack is non-apoptotic (Mittler and Lam, 1996), but again DNA fragmentation can be detected (Ryerson and Heath, 1996; Wang, Bostock, and Gilchrist, 1996). The formation of root aerenchyma by cell lysis has been described as PCD (Drew, 1997), and in *Zea mays* (Campbell and Drew, 1983) and *Oryza sativa* (Webb and Jackson, 1986) this process appears to be non-apoptotic.

In *Z. mays*, cell lysis in roots has been related to the orientation of cortical microtubules (CMT) (Baluska et al., 1993). After an increase in ethylene concentration, ordered arrays of CMTs rapidly become disorganized in certain cells of the root cortex and it is hypothesized that this change may correlate with cell lysis (Baluska et al., 1993). The orientation of cortical CMT arrays can also be related to the direction of cell expansion because layers of cell wall are laid down in the same orientation as the CMT arrays (Barlow and Parker, 1996). In expanding root cells, the orientation of CMT arrays typically range from perpendicular to the root axis to diagonal to the root axis (Williamson, 1991). However, orientation of CMT arrays can be rapidly altered by

external signals such as hormones or wounding, leading to changes in patterns of cell expansion (Hush and Overall, 1996; Wymer and Lloyd, 1996).

In this chapter, the structure of root cortex cells in *S. lancifolia* was examined at different stages of aerenchyma development. The structure of root cortex cells in *Z. mays* and *O. sativa* was also examined to compare with *S. lancifolia*. Specific objectives were to characterize the process of aerenchyma formation by cell lysis in different species and to compare features if cell lysis to known characteristics of PCD. Cell death in the three species was not apoptotic, and there were differences in cell morphology during cell lysis among the species. In roots of *S. lancifolia*, radial expansion of unusual diaphragm cells may play a central role in gas space development.

MATERIALS AND METHODS

Plant Tissue Preparation

Sagittaria lancifolia plants (Horticultural Systems, Parrish, FL) were grown in aerated one-quarter strength Hoagland's solution (Epstein, 1972) under controlled temperatures and photoperiod in a growth chamber (Environmental Growth Chambers, Chagrin Falls, OH). Seeds of *Z. mays* and *O. sativa* cv. Labell were germinated on moist vermiculite in a growth chamber and two-week old seedlings were transferred to modified Hoagland's solution (Yoshida et al., 1976; Drew, Jackson, and Giffard, 1979). All plants were grown at a 12 h photoperiod with 115 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation at the base of the petioles with day and night temperatures of 32 ° and 25 °C, respectively. Plants were grown in hydroponic solutions that were either

constantly aerated (to 9-10 ppm of O_2) or bubbled with N_2 gas twice daily for 30 min (to 3-5 ppm of O_2) for at least one week prior to sampling.

Cell Structure in *S. lancifolia*, *Z. mays*, and *O. sativa*

Sagittaria lancifolia root tissue was sampled from different developmental stages along the length of similarly-sized roots. *Zea mays* and *O. sativa* root tissue was sampled from late developmental stages where cell lysis occurred. Tissue pieces were fixed in 1 % para-formaldehyde, 2 % glutaraldehyde, 0.05 M sodium cacodylate buffer (pH 7.2), 0.05 % calcium chloride, and 2 % sucrose, under vacuum, overnight at room temperature. The tissue was rinsed in sodium cacodylate buffer with sucrose and post-fixed in 4 % osmium tetraoxide. The tissue was rinsed in buffer, stained in 0.5 % uranyl acetate and dehydrated in a graded ethanol series. LR White medium grade resin (Electron Microscopy Sciences, Fort Washington, PA) was slowly substituted for the ethanol. Polymerization was at 58-60 °C for 24 h.

One μm thick sections were cut perpendicular to the root axis with a microtome (Sorvall MT-2 "Porter Blum" Ultra-Microtome, Norwalk, CT) and placed on collodoin-coated grids. These cross sections from roots were stained with lead citrate and cortex cells examined with a transmission electron microscope ("JEOL JEM 100 CX").

Orientation of CMT arrays in *S. lancifolia*

Sagittaria lancifolia plants were grown as described above. Sections of roots (3-4 mm long) representing different stages of root development were fixed overnight, at 4 °C, in 3.7 % formaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 2 mM $MgCl_2$, 10 mM EGTA), pH 6.9, with 5 % dimethylsulfoxide (DMSO). After rinsing in

the buffer, longitudinal sections (70 μm thick) were cut with a vibratome. The sections were placed on a coverslip and covered with an agarose-gelatin film (Brown and Lemmon, 1995). Cell walls were initially digested in an aqueous solution of 1 % cellulase, 1.5 % β-glucuronidase, and 1 % glucose for 25 min, and then permeabilized in 1 % Triton X-100 in PHEM for five min. Sections were incubated in an anti-tubulin primary antibody (rat anti-yeast tubulin, Accurate Chemical and Scientific Corporation, Westbury, NY) for one hour. Sections were then incubated overnight, in the dark, with a secondary antibody (goat anti-rat IgG conjugated to FITC (fluorescein-5-isothiocyanate), Accurate Chemical and Scientific Corporation, Westbury, NY). Sections were counterstained with 0.01 % propidium iodide for ten seconds and mounted with 20 % Mowiol 4.88 with the anti-fade agent *p*-phenylenediamine. Sections were examined with a Bio-Rad MRC-600 confocal laser scanning microscope (Bio-Rad Laboratories, Hercules, CA).

DNA Fragmentation in *S. lancifolia*

Sagittaria lancifolia root tissue from different developmental stages was fixed and sectioned as described for CMT arrays (above). Pre-treatment with proteinase K (10 μg/ml for ten min) was followed by application of the TdT-mediated nick end labeling (TUNEL) assay using an *in situ* cell death detection kit with an FITC tag (Boehringer Mannheim, Indianapolis, IN). Pre-treatment variations included proteinase K at 5 μg/ml for ten min or no application of proteinase K, application of Triton X-100 (1 % for five min) after the proteinase K, and application of cell wall degrading enzymes prior to the assay (as in the CMT array methods above). The TUNEL assay

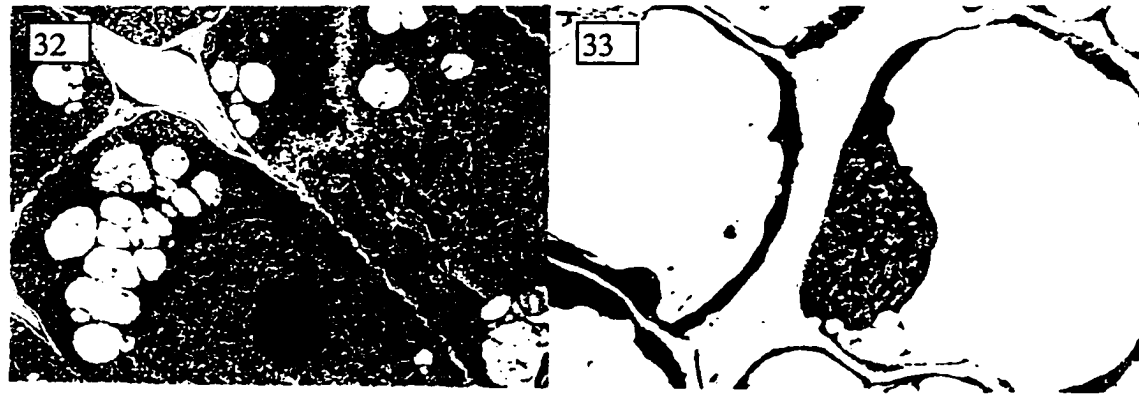
was applied in concentrations outlined in the kit instructions, as well as at half concentration. The sections were counterstained with 0.01 % propidium iodide for ten seconds to visualize nuclei and mounted in a drop of 20 % Mowiol 4.88 in PBS buffer, pH 8.5, containing *p*-phenylenediamine as an anti-fade agent. Sections were examined for propidium iodide and FITC fluorescence with a Bio-Rad confocal laser scanning microscope (Bio-Rad Laboratories, Hercules, CA).

Root sections were analyzed for "DNA laddering", the enzymatic cleavage of DNA into specific-sized fragments. For these measurements, four roots, approximately 8 cm in length were cut into four sections, representing different developmental stages. All of the sections for a given developmental stage were pooled and ground in liquid N_2. DNA was extracted (Gentra Systems, Inc., Puregene DNA Isolation Kit, Minneapolis, MN) from each pooled sample and 30 μL of extract was loaded on a 1 % agarose gel and electrophoresed for 18 h at 35 V. Ethidium bromide was used to visualize DNA and molecular weights of bands were estimated to molecular markers from a *Hin*d III Lambda Digest.

RESULTS

Cell Structure in *Sagittaria lancifolia*

Just behind the root tip, cortex cells of *S. lancifolia* had nuclei with intact membranes, smooth surfaces, and chromatin that appeared to be dispersed normally. These cells also had distinct ribosomes, dictyosomes, mitochondria, and intact membranes (Fig. 32). Cell lysis was not observed at this stage and the preservation of features indicated good fixation. These cells had larger amounts of cytoplasm and



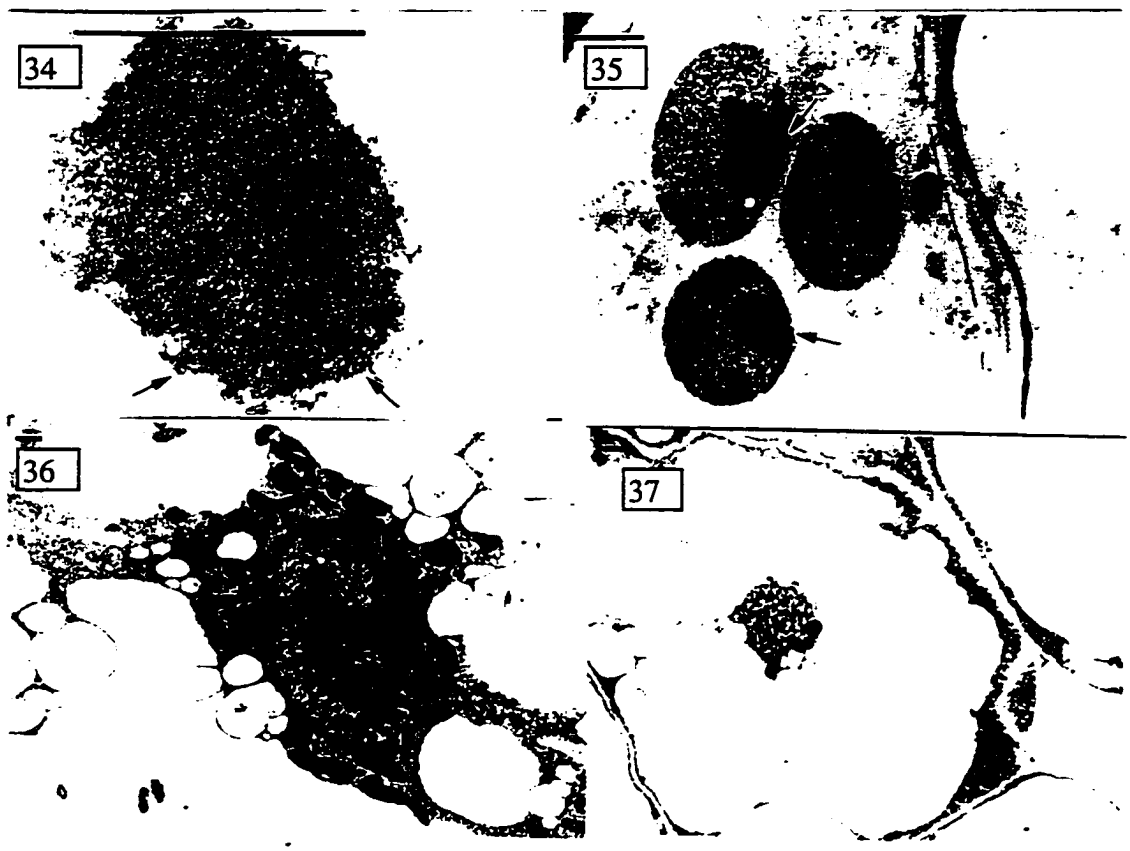
Figures 32-33. Root cross sections of *S. lancifolia*, showing cells of the cortex region. 32. Cells directly behind the root tip with intact structure, large cytoplasmic area, and small vacuoles. 33. Cells farther from the root tip with large vacuole and cytoplasm around the periphery of the cell. Bars = 1 μm.

smaller vacuoles than cells 10 mm or more behind the root tip where large vacuoles restricted cytoplasm to the cell periphery (Fig. 33).

Abnormal nuclei appeared to be the first observable step in cell lysis when young and old stages of root cortex were compared. Nuclear membranes appeared to deteriorate (Fig. 34), nuclei began to fragment (Figs. 35, 36), and nuclei appeared intermixed with vacuoles (Fig. 37). These changes in nuclear structure were found in cortex cells from just behind the root tip to up to 2 cm behind the root tip (depending on the root examined).

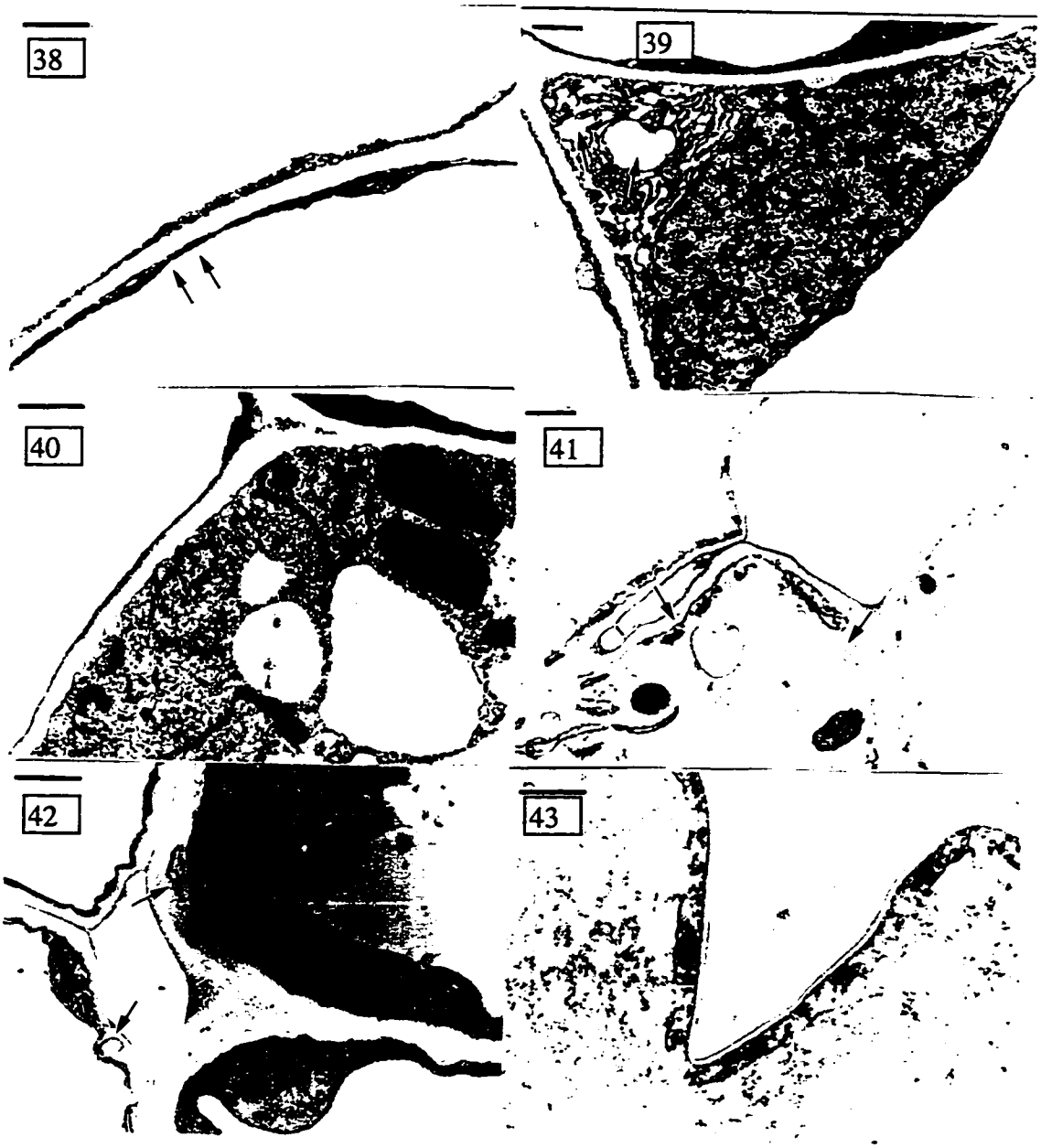
Abnormalities in cytoplasmic contents were first observed at about 2 cm behind the root tip. These changes included condensation of the cytoplasm (Fig. 38), electron-lucent (clear) regions in the cytoplasm (Figs. 39, 40), and crenulation of the plasma membrane (Fig. 41). Particulate matter occasionally appeared between the plasma membrane and cell wall (Figs. 41, 42). In some cells, the tonoplast of the cell had disintegrated and altered the appearance of the cytoplasm (Figs. 41, 43).

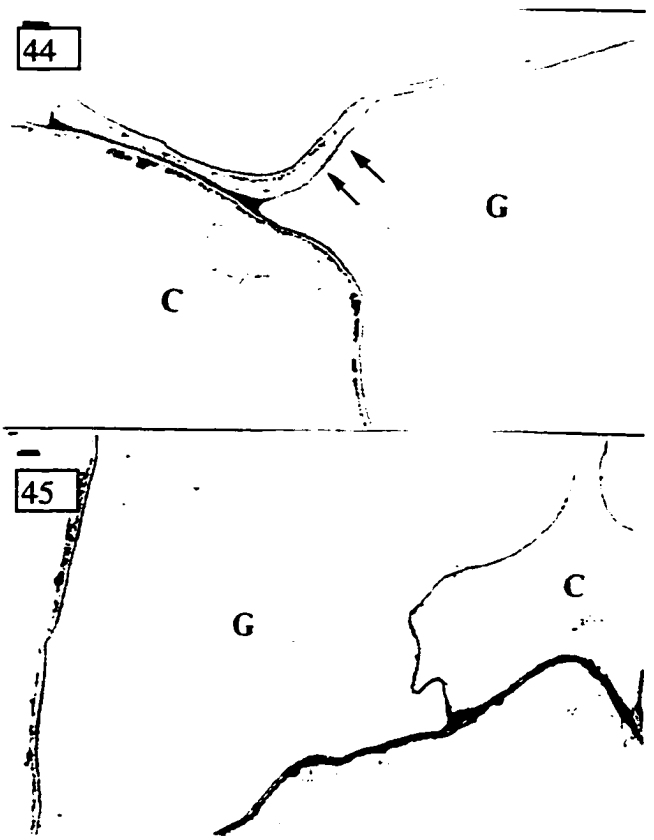
In the cortex 3-4 cm behind the root tip, the cells were devoid of contents except for some occasional, diffuse cytoplasm. Organelles, especially the nuclei, were rarely seen in these cortex cells. Many of the cortex cells were collapsing, or had collapsed, creating the gas spaces characteristic of aerenchyma tissue (Fig. 44). The cell wall had not deteriorated in collapsed cells (Fig. 44). When cells collapsed they became detached from neighboring radial files of cells in the cortex. However, collapsed cells within the same file remained attached to one another (Fig. 45).



Figures 34-37. Abnormal nuclear structures in cross sections of cortex cells in *S. lancifolia*. 34. Nucleus with disintegrated nuclear membrane, but still with nuclear pores (arrows). 35. Nucleus with condensed chromatin (arrows). 36. Nucleus breaking apart in the vacuole. 37. Nucleus inside the vacuole. Bars = 1 μm.

Figures 38-43. Changes in the cytoplasm and membranes of cross sections of cortex cells in *S. lancifolia*. 38. Condensation of the cytoplasm against the edges of the cell (arrows). 39. Numerous electron-lucent regions (arrows) within the cytoplasm. 40. Large electron-lucent regions in the cytoplasm with some material inside. 41. Crenulation of the plasma membrane (arrows) in a cell with a disintegrated tonoplast. 42. Cell with material outside of the plasma membrane (arrows). 43. Cell with disintegrated tonoplast, diluted cytoplasm, and granular material inside of the vacuole. Bars = 1 μm.





Figures 44-45. Cross sections of cortex cells during late stages of cell lysis in *S. lancifolia*. 44. Completely lysed cell (arrows) next to a degraded, but not collapsed, neighboring cell. 45. Two adjacent files of cells with collapsed cell detached from the neighboring cell, but still attached to its radial file neighbor. Bars = 1 μm. C, cell; G, gas space.

Cell Structure in *Z. mays* and *O. sativa*

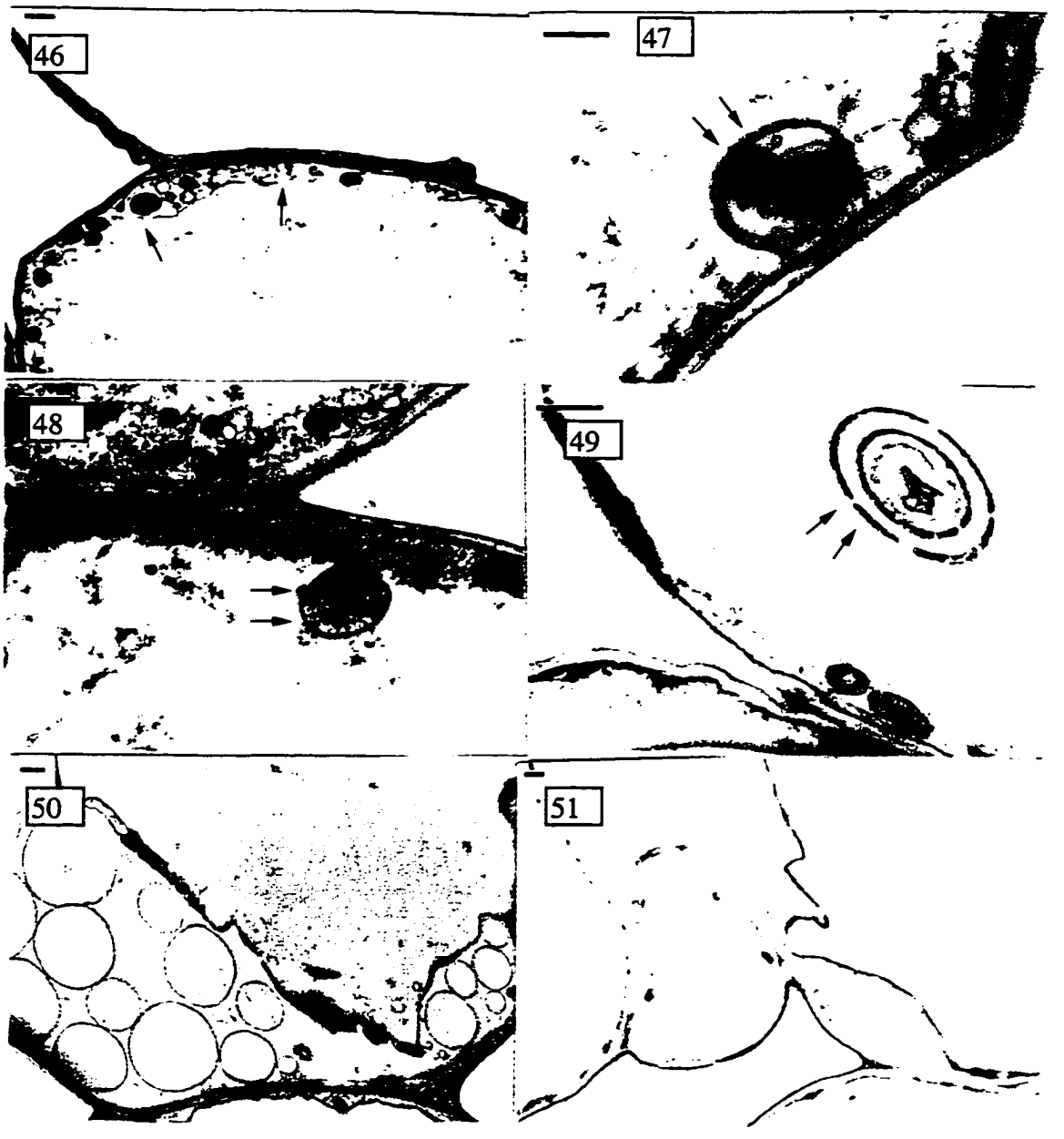
Nuclei were not observed in cortex cells undergoing cell lysis in *Z. mays*. The vacuoles of many cells in the cortex contained granular material and tonoplast membranes of these cells were rarely intact (Fig. 46). Despite the partial dissolution of the cytoplasm, organelles were still discernible around the periphery of these cells (Fig. 46). Concentric circles of membranes were occasionally observed in the cells (Fig. 47), and inward blebbing of cytoplasm into the vacuole was observed (Fig. 48). After disintegration of the cytoplasm, cells collapsed but retained their cell wall (Fig. 46), as observed in *S. lancifolia* (Fig. 44).

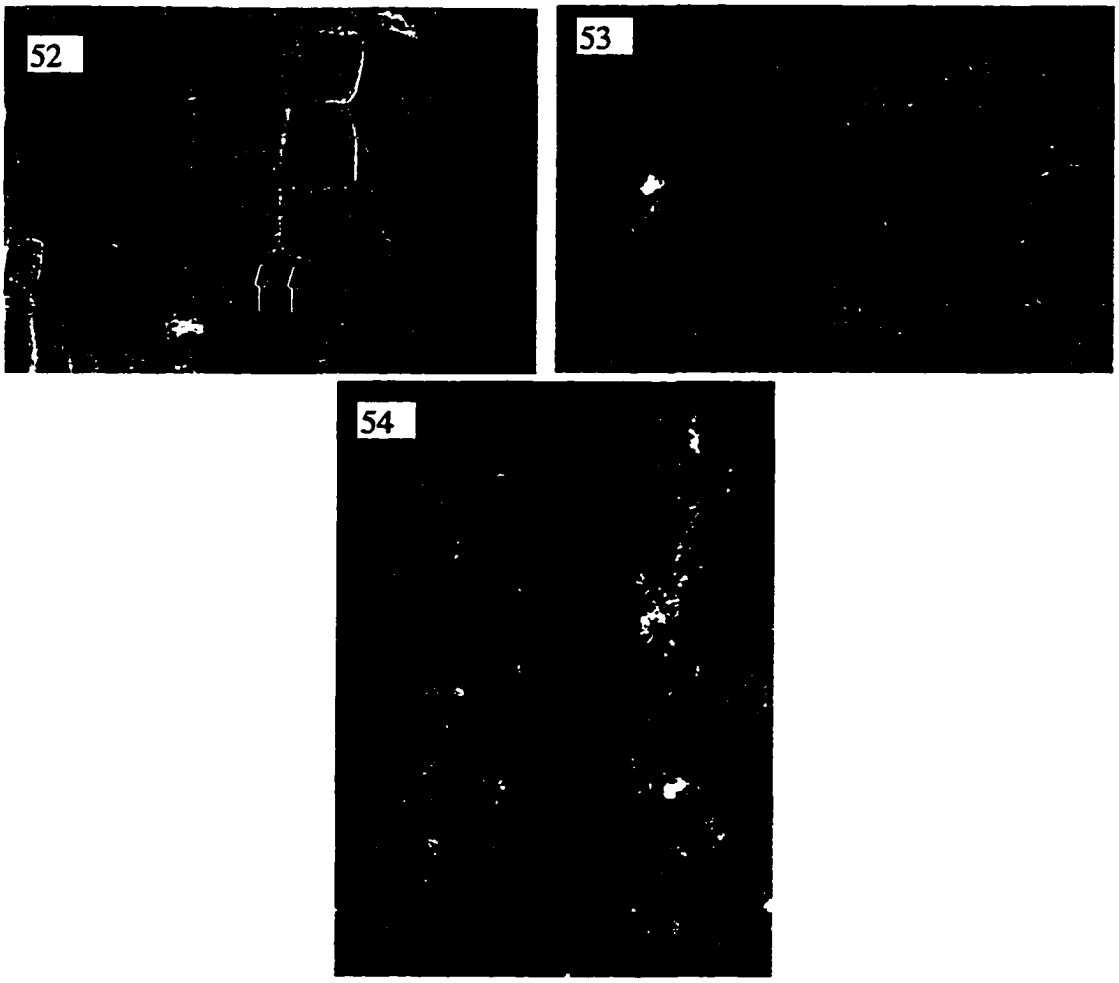
Nuclei were also not observed in lysing cortex cells of *O. sativa*. Often at this late developmental stage, tonoplast membranes had disintegrated and organelles were swollen and distorted (Fig. 49). Concentric circles of membranes were also observed in *O. sativa*, as in *Z. mays* (Fig. 49). There were empty, membrane-bound structures or granular material instead of recognizable organelles in some cells (Figs. 50, 51). Despite the advanced stages of membrane and organellar disintegration, none of the cortex cells from *O. sativa* were observed to be completely collapsed as in *S. lancifolia* and *Z. mays*.

Orientation of CMT arrays in *S. lancifolia*

The orientation of CMT arrays was characterized by immuno-fluorescence to determine if orientation predicted which cells would lyse during root aerenchyma development. Close to the root tip, CMT arrays of cortex cells were randomly oriented (Fig. 52). Occasional cellular divisions parallel to the root axis (t-divisions) produced new files of cortex cells (Fig. 52). Behind the root tip, orientation of the CMT arrays in

Figures 46-51. Late stages of cell lysis in cross sections of cortex cells of *Z. mays* and *O. sativa*. 46. Degraded cell of *Z. mays* with disintegrated tonoplast membrane (arrows), granular material in vacuole, and adjoining collapsed cell. Organelles appear dilated. 47. Cell of *Z. mays* with concentric circles of membrane (arrows) and granular tonoplast. 48. Cell of *Z. mays* with disintegrated tonoplast, degraded organelles, and internal blebbing into vacuole (arrows). 49. Cell of *O. sativa* with disintegrated tonoplast and concentric circle of membranes (arrows). 50. Cell of *O. sativa* with empty vesicles in the cytoplasm. 51. Two cells of *O. sativa* with granular material in the cytoplasm. Bars = 1 μm.





Figures 52-54. Orientation of cortical microtubule arrays in longitudinal sections of *S. lancifolia* at different developmental stages. 52. Cells close to the meristem with randomly oriented microtubules and a t-division (arrows) to create a new file. 53. Cells slightly farther back in regular files with CMT arrays oriented perpendicular to the axis of the root. 54. Cells far from the root tip that have elongated cortex cells and CMT arrays oriented parallel to the root axis.

cortex cells became perpendicular to the root axis (Fig. 53). At 2-3 cm behind the root tip, cortex cells had elongated significantly and the CMT arrays of these cells were oriented diagonally (Fig. 54).

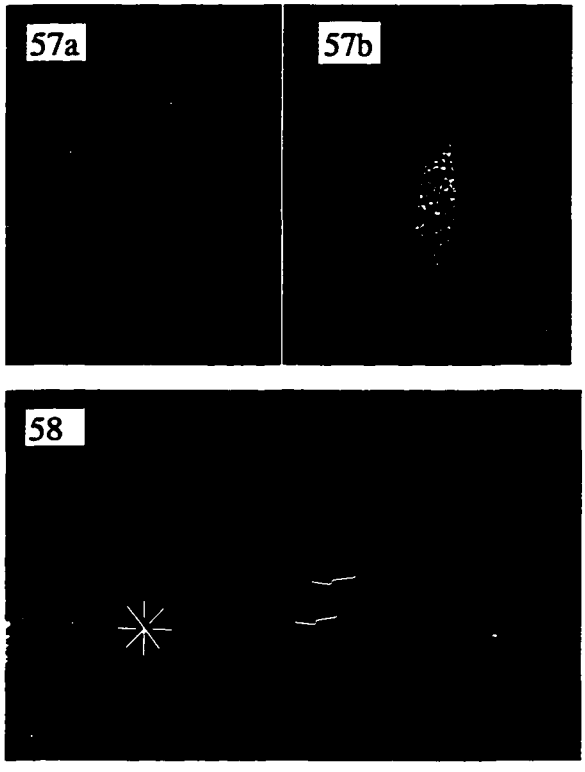
In longitudinal sections of mature root tissue, layers of cells were observed perpendicular to the root axis. These layers of what will be termed diaphragm cells were separated by cortex cells. The diaphragm cells differed from other cells in the cortex in that they were alive in the mature aerenchyma tissue and had thin, cellular extensions at their peripheries (Fig. 55). The CMT arrays were unusual in these diaphragm cells in that their orientation was parallel to the root axis (Fig. 56).

DNA Fragmentation in *S. lancifolia*

Nicked DNA, a hallmark of apoptosis, was not detected in root cortex cells of *S. lancifolia* by the TUNEL assay, even though the basic assay protocol was varied in a number of ways (see Materials and Methods). Occasionally, nuclei sheared from the tissue during sectioning showed what appeared to be positive labeling by TUNEL (Fig. 57). Fluorescence in such nuclei (Fig. 57B) was much brighter than in nuclei labeled by propidium iodide (Fig. 57A). Some nuclei of epidermal cells close to the root tip (Fig. 58) also appeared to be positively labeled by the TUNEL assay, while cortex cells in the same section were not.

DNA extracted from different developmental stages of *S. lancifolia* roots did not produce "DNA laddering". The DNA from each stage was at least 23 kb (Fig. 59).

Figures 55-56. Root diaphragm cells in *S. lancifolia*. 55. View of diaphragm cells in a root cross section showing thin cellular extensions (arrows) at the edges of the cells. 56. Longitudinal sections of mature diaphragm cells with CMT arrays oriented parallel to the axis of the root.



Figures 57-58. Results of the application of the TUNEL procedure to longitudinal sections of *S. lancifolia* roots. 57. Nucleus sheared from the tissue during sectioning showing propidium iodide fluorescence in the left frame (A) and an even stronger FITC (TUNEL label) fluorescence in the right frame (B). 58. Example of a section where nuclei of epidermal cells (arrows) appear to be labeled by the TUNEL assay while cortex cells (star) show no TUNEL label.

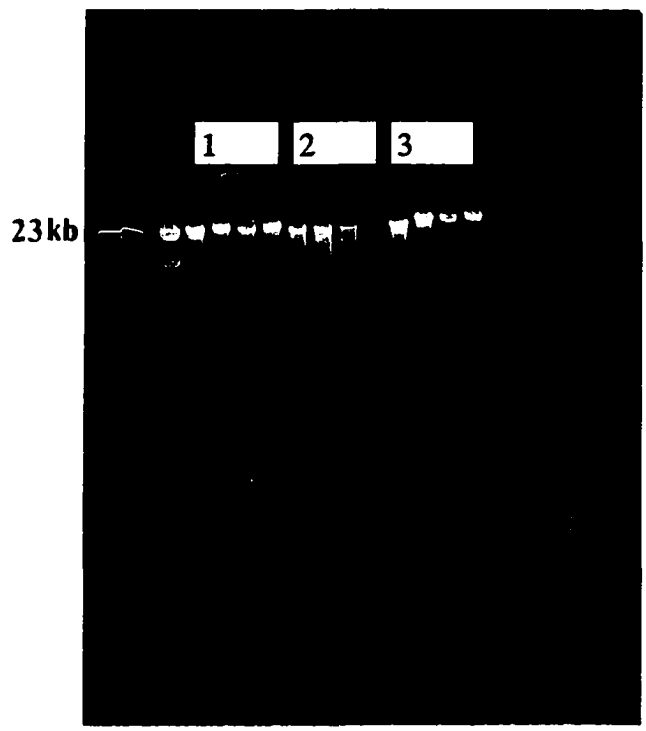


Figure 59. Agarose gel of the DNA of three sets of roots (labeled 1-3) extracted from four different stages of development (left lane in each set is the youngest and right lane the oldest) with *Hin*d III lambda digest molecular markers in the far left lane.

DISCUSSION

As root cortex cells of *S. lancifolia* lyse to form aerenchyma, they exhibit a unique sequence of morphological changes. These changes are not consistent with previous descriptions of necrosis or apoptosis (Kerr, Wyllie, and Currie, 1972). The sequence of morphological changes during death of cortex cells of *S. lancifolia* started with changes in the nucleus, including clumping of the chromatin, apparent fragmentation, disruption of the nuclear membrane, and appearance in the vacuole. These nuclear changes were followed at later stages by plasma membrane crenulation, the formation of electron-lucent regions in the cytoplasm, tonoplast disintegration, organellar swelling and disruption, the removal of cytoplasmic contents, and the final collapse of the cell (Table 2).

The changes in nuclear structure, condensation of the cytoplasm, and maintenance of organelle integrity until late in the cell death process observed in *S. lancifolia* are also characteristic of cells undergoing apoptosis (Kerr, Wyllie, and Currie, 1972). However, apoptotic characteristics such as pycnosis of the nucleus and plasma membrane blebbing with the subsequent production of apoptotic bodies were not observed in lysing cells of *S. lancifolia*. The observations were also not consistent with definitions of cell necrosis because mitochondria were not the initial target of cell destruction and cells did not swell (Kerr, Wyllie, and Currie, 1972). While the death of cortex cells in *S. lancifolia* does not include the total list of characteristics for apoptosis, the initial changes in nuclear structure and the delayed disintegration of mitochondria are indicative of PCD.

Table 2: Changes in cell morphology of cortex cells of *S. lancifolia*.

Developmental Timing	Observation
"Early"	Chromatin Clumping Nuclear Fragmentation Nuclear Membrane Disruption Nucleus Intermixed in Vacuole
"Middle"	Plasma Membrane Crenulation Electron-Lucent Regions in the Cytoplasm Tonoplast Disintegration Swelling and Disruption of the Organelles Cytoplasmic Contents Disintegrate
"Late"	Cell Collapse

Apoptotic characteristics are considered the hallmark of PCD by many reviewers of PCD in plants (Greenberg, 1996; Havel and Durzan, 1996). However, most reports of plant PCD do not fit apoptosis (Mittler and Lam, 1996; Groover et al., 1997). The presence of some of the features of apoptosis in plant cells seems illogical. Apoptosis involves an outward blebbing of the plasma membrane and phagocytosis of cell contents by neighboring cells. This would seem unlikely in plant cells because of presence of cell walls. In animal cells certain types of non-apoptotic PCD (Clarke, 1990) could potentially be more applicable to plant systems than apoptosis. Morphological features of cell death in *S. lancifolia* resemble autophagic degeneration as described by Clarke (1990). Autophagic degeneration involves an internal blebbing of cell contents into vacuoles which is more consistent for a cell with a large vacuole and a wall.

Changes in cell structure during cell lysis in *S. lancifolia*, *Z. mays* and *O. sativa* were similar, but did vary. Differences among the three species centered on changes in membrane structure. The disruption of the tonoplast occurred at an earlier developmental stage of cell lysis in *Z. mays* than in *S. lancifolia* or *O. sativa.* This difference between *Z. mays* and *O. sativa* was reported previously (Campbell and Drew, 1983; Webb and Jackson, 1986). Concentric circles of membranes were observed in lysing cells of *Z. mays* and *O. sativa*, but not in *S. lancifolia*. Similar structures have been observed in cells from *Pisum sativum* roots growing at low O_2 concentrations (Davies et al., 1987). Davies et al. (1987) hypothesized these structures represent a rearrangement of endoplasmic reticulum that occurs when energy charge in the cell drops below a minimum value needed to maintain the more typical arrangement found

in healthy cells. If this hypothesis is correct, the fact that these concentric membrane structures were not observed in *S. lancifolia* may mean these cells are better able to maintain cellular energy charge, perhaps by maintaining mitochondria structure longer than *Z. mays* or *O. sativa*. Differences among the three species in membrane alterations may reflect differences in their mechanisms of cell lysis.

The orientation of CMT arrays in most of the cortex cells in *S. lancifolia* roots was consistent with previous reports from other species of CMT orientation in developing root cells (Williamson, 1991; Fig. 60). Ordered CMT arrays perpendicular to the root axis were observed in cells close to the root tip (part A, Fig. 60), but as cells expanded the CMT arrays became diagonal (part B, Fig. 60). It has been hypothesized that CMTs become disorganized before root cortex cells undergo lysis in *Z. mays* (Baluska et al., 1993). In *S. lancifolia*, CMTs were almost always observed in ordered arrays, even five to six centimeters from the root tip, but some disorganization was observed as cortex cells separated prior to lysis (part C, D, Fig. 60). *S. lancifolia* roots possess unique diaphragm cells with CMT arrays oriented parallel to the root axis (part C, Fig. 60), an orientation that is rare during root development (Williamson, 1991). This orientation predicts diaphragm cells would expand in a direction that is perpendicular to the plane of the root axis. The separation of the files of cells in the root cortex of *S. lancifolia* prior to lysis (Chapter 2) could be the result of radial expansion of the diaphragm cells.

DNA nicking and systematic degradation of DNA prior to cell lysis to form root aerenchyma was not observed in *S. lancifolia*. There have been examples of PCD in

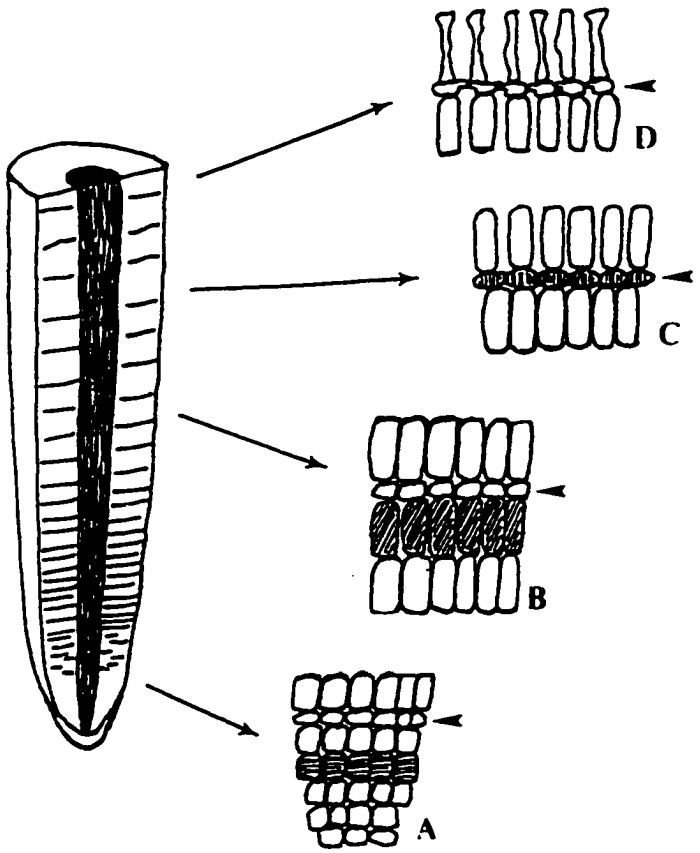


Figure 60. Representation of diaphragm layers in longitudinal view of a root of *S. lancifolia*. (A) Close to the meristem with CMT arrays (hatched lines) of cortex cells perpendicular to the root axis. (B) Beyond the region of elongation, with CMT arrays of cortex cells diagonal to the root axis. (C) CMT arrays parallel to the root axis in diaphragm cells. (D) Mature region with lysis of the cortex cells and intact diaphragm structure. Arrows on right point to diaphragm layers.

animal cells that lack this specific type of DNA fragmentation (Schwartz et al., 1993). In plant systems, nicked DNA has been found with the TUNEL assay, but there have been instances where DNA laddering was not found even when TUNEL results were positive (Mittler and Lam, 1996). Lack of DNA laddering in some plants may suggest that the progression of DNA fragmentation may differ between plants and animals.

Structural changes of cells during lysis to form root aerenchyma in *S. lancifolia*, *Z. mays* and *O. sativa* suggest these systems are examples of PCD. Comparisons among these species, however, indicate that cell lysis to form aerenchyma can be different, just as the regulation of root aerenchyma formation differs among the species.

CHAPTER 5

CONCLUSIONS

This dissertation has focused on aerenchyma development in *Sagittaria lancifolia*, a marsh species adapted to waterlogged environments. Aerenchyma formation in this species was compared to this process in *Zea mays* and *Oryza sativa*. Differences in aerenchyma formation between the roots and petioles in *S. lancifolia* (Chapter 2) and in the formation of root aerenchyma by cell lysis among *S. lancifolia*, *Z. mays*, and *O. sativa* have been shown (Chapter 3, 4).

There is constitutive formation of aerenchyma by cell separation in petioles of *S. lancifolia* and the relative volume of aerenchyma in petioles is similar in plants grown at different O_2 concentrations in the root zone. In roots of *S. lancifolia*, *Z. mays* and *O. sativa*, aerenchyma tissue is formed by cell lysis, but the formation in each species responds differently to normal and low O_2 concentrations in the root zone. In *S. lancifolia* and *O. sativa*, root aerenchyma forms constitutively, but the relative volume of aerenchyma increases in *S. lancifolia* plants grown in low root zone O_2 concentration, but not in *O. sativa*. There is cell lysis in roots of *Z. mays* grown at low O_2 concentration in the root zone, but cell lysis does not occur at normal O_2 concentration. Specific differences in membrane disintegration characteristics were observed among the three species. In the roots of *S. lancifolia*, the radial expansion of diaphragm cells may play a role in the formation of root aerenchyma by separating cells of the cortex prior to cell lysis. This function is similar to the expansion of diaphragm cells in the petioles to create gas spaces.

Control of Root Aerenchyma Formation By Cell Lysis

A general model explaining cell lysis must account for the observed differences among the three species. Major questions that must be addressed include: How is selective lysis among the cells of the cortex achieved? Is constitutive and environmental induction of aerenchyma formation regulated by the same set of cellular events? Why do cells from different species exhibit different morphological characteristics during cell death? A framework for such a model must first be constructed before these questions can be answered.

The basis of the model proposed here is the finding in this dissertation that a decrease in root zone O_2 concentration can produce different responses in cells. Since ethylene concentration is closely correlated with root zone O_2 concentration and aerenchyma formation in *Z. mays* (He, Morgan, and Drew, 1992), the model assumes ethylene is the signal by which cells "sense" O_2 concentration. A family of putative ethylene receptors thought to be localized in cell membranes has been identified in *Arabidopsis* (Kieber, 1997). Analysis of the genes encoding these putative receptors indicates that the receptors are present as dimers and act as kinases. In the absence of ethylene, the kinase activity of the receptor phosphorylates CTR1 which when phosphorylated inhibits any further downstream events in the signal transduction pathway. When ethylene binds to the ethylene receptor, the kinase activity of the receptor is blocked, CTR1 becomes inactive, and a cascade of downstream events occur (Kieber, 1997). In the case of aerenchyma development, it is postulated that the ethylene signal would activate a sequence of steps including initiation of PCD and aerenchyma formation. Addition and inhibition of possible signal transduction elements

such as inositol phospholipids, Ca-calmodulin, protein kinases, G-proteins, cytosolic calcium, and protein phosphatases affect cellulase activity and cell lysis in roots of *Z. mays* (He, Morgan, and Drew, 1996). A sequence of events relating O_2 concentration in the root zone and the formation of aerenchyma in different types of cells is shown in Figure 61 and described below.

Lysis of specific cells in the root cortex could be due to differences in sensitivity to the ethylene signal. Variation in cell response to a signal may be explained by an epigenetic control of sensitivity to the signal (Trewavas and Mahlo, 1997). Epigenetic refers to the effect of multiple gene products on signal transduction in a cell. Cells with different combinations (and/or concentrations) of gene products may respond differently to a specific signal. Cellular differences in expression of genes for signal receptors or combinations of different types of signal receptors could produce selective lysis of certain cortex cells during aerenchyma formation. Therefore, the biochemical and physiological response may be similar among cells, but the concentration necessary to trigger these responses would differ. For example, brief exposure to ethylene in *Z. mays* roots may cause a change in the orientation of CMT arrays in certain cortex cells but not in others (Baluska et al., 1993). In the model shown in Figure 61, cell A and C might both possess the pathway that responds to a signal such as ethylene, but only A would have the sensitivity (related to the number of receptors per cell) to be triggered by physiological concentration of the signal.

There are also differences among species in the environmental conditions that trigger cell lysis. Cell lysis in *Z. mays* only occurs in response to environmental stress

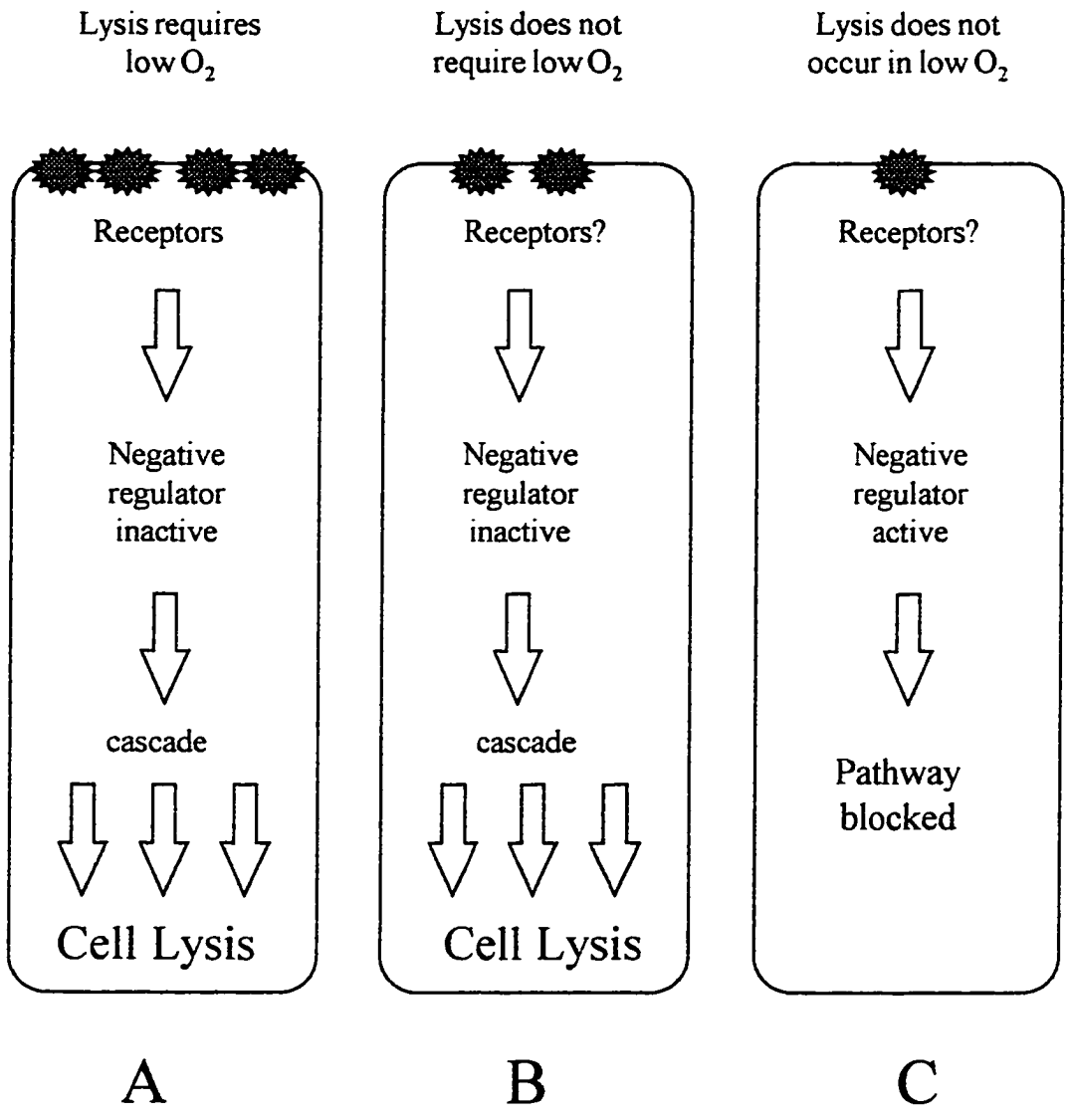


Figure 61. Model of differential regulation of cell lysis. A represents cortex cells from *S. lancifolia* and *Z. mays* that lyse when the concentration of O_2 in the root zone is low. B represents cortex cells from *S. lancifolia* and *O. sativa* that do not require a low concentration of O_2 for cell lysis to occur. C represents root cortex cells in all three species that do not lyse.

(Atwell, Drew, and Jackson, 1988), but *O. sativa* and *S. lancifolia* appear to form aerenchyma under all conditions. Lysis in *Z. mays* can be explained by activation of the signal transduction pathway (Fig. 61, cell A) in only certain cells of the cortex that are sensitive to a signal produced by decreases in O_2. Cell C of Figure 61 would represent a cortex cell in *Z. mays* that would not lyse in response to low O_2 unless its sensitivity to the signal increased. This cell type would also be representative of cortex cells in *S. lancifolia* that lyse when O_2 concentration decreases. In *S. lancifolia* and *O. sativa*, constitutive aerenchyma formation may be explained by certain cells of the cortex having a signal transduction pathway that is like the ethylene stimulated pathway, but either lacks the negative regulation element or has one that is never active (Fig. 61, cell B). Cells lacking negative regulation of the pathway would not require the signal to trigger the response, thus these species would appear insensitive to changes in O_2 or the signal. For example, there are mutants of *Arabidopsis* that produce the classic triple response to ethylene, without ethylene in the system, because of a lack of negative regulation of the pathway (Kieber et al., 1993).

There were differences among species in the changes in cell morphology that occurred during cell lysis to form root aerenchyma. This could be explained by assuming a split in the pathway after the transduction of the initial signal. This is represented in Figure 61 by multiple arrows following the cascade of signals. Variations in the morphology of cells during PCD have been hypothesized to exist because of more than one sequence of steps directing cell death (Greenberg, 1996; Jones and Dangl, 1996). So another explanation would be that *S. lancifolia*, *O. sativa*, and *Z.*

mays may each have different and unique pathways that lead to cell lysis and formation of root aerenchyma downstream from the negative regulator.

The model presented in Figure 61 assumes that there is a single signal transduction cascade for all types of aerenchyma formation. However, the possibility of different sets of cellular events directing different types of aerenchyma formation cannot be ruled out.

Plants, Apoptosis, and Programmed Cell Death

The morphological criteria for apoptotic cell death were defined for animal cells, and many of these apoptotic characteristics are not observed in examples of plant PCD. Apoptotic bodies, a salient feature of apoptosis, have not been clearly observed in plant PCD. The outward plasma membrane "blebbing" required for formation of the apoptotic bodies may be impossible given the limitations of a plant cell wall. Internal blebbing into vacuoles of plants has been observed and may be a more likely feature representing disintegration of cell contents. In this study, particulate material as well as nuclei were observed inside the vacuoles of cortex cells (Chapter 4). In plants, the morphological characteristics of cells undergoing PCD appear not to be apoptotic, but instead may represent a morphology unique to plant PCD.

Evolution of Aerenchyma Formation

In primitive land plants that normally possessed no gas spaces, a plant with a mutation in its pathogenic response pathway may have had random disintegration of cells in the root cortex in response to stresses such as waterlogging. This process may not have produced well organized or necessarily interlinked gas spaces, but would have

facilitated gas transport to roots under waterlogged conditions. This response may be similar to the gas space formation observed in *Z. mays* roots exposed to waterlogging.

Through selection, the production of gas spaces in the roots may have become widespread throughout the cortex, with interlinked lacunae. Selection may have resulted in species with a constitutively-expressed pathway of gas space production. This constitutive production would have benefited plants in frequently waterlogged habitats because they would be pre-adapted for O_2-limited conditions. This may be similar to the response observed in *O. sativa* roots.

With further selection for more efficient aerenchyma structure, diaphragm cells may have provided a means to regulate the spacing of lacunae. The organization of aerenchyma in *S. lancifolia* roots would fit this idea. Diaphragms are also observed in the shoots of a number of species, providing a continuous, well organized pathway for O_2 transport to the root zone.

This dissertation has characterized diversity in the formation of gas spaces in species possessing and lacking adaptations to waterlogging. Aerenchyma formation by cell lysis and by cell separation can exist in one species, and the characteristics of formation of root aerenchyma can vary among species. Future studies are needed to better document the diversity in aerenchyma formation among different species and habitats.

Differences in aerenchyma formation among species can be used to model how cell death and cell separation occur in different species. Despite the apparent differences among species in the initiation of cell lysis, the process appears to be remarkably well conserved, as evidenced by the general similarity among the three

species in cell morphology during cell death. Information about changes in cell structure during cell lysis and the cellular control of this process will be important to the continued research on the diversity of PCD in plants.

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APPENDIX

LOUISIANA STATE UNIVERSITY
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Department of Plant Biology

June 25, 1997

Dr. Karl J. Niklas
Editor, The American Journal of Botany
Section of Plant Biology
Cornell University
Ithaca, NY 14853-5908

Dear Dr. Niklas:

I am writing to obtain permission to reproduce text and data from the following article in my dissertation:

> Schussler, E. E., and D. J. Longstreth. 1996. Aerenchyma develops by cell lysis in roots and cell separation in leaf petioles of *Sagittaria lancifolia* (Alismataceae). American Journal of Botany 83(10): 1266-1273.

I am currently a candidate for the Ph.D. Degree in the Department of Plant Biology at Louisiana State University. I will be graduating in December of this year, so permission needs to be granted by the end of September at the latest.

Thank you very much for your assistance.

Sincerely,

Elisabeth Schussler

Elisabeth Schussler

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2 July 1997

Ms. Elisabeth Schussler
Department of Plant Biology
Louisiana State University
Baton Rouge, LA 70803-1705
FAX: 504-388-8459

Dear Ms. Schussler:

This letter provides you with authorization from the *American Journal of Botany* to use any material necessary from:

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in your Ph. D. disssertation to be submitted to the Department of Plant Biology at Louisiana State University.

Sincerely,

Karl Niklas

Karl Niklas (Editor-in-Chief)

VITA

Elisabeth Ellen Schussler was born October 21, 1970, in West Lafayette, Indiana. She lived in Ohio, Delaware, and Mississippi before returning to Delaware for high school. She graduated from Newark High School in 1988 and entered Vanderbilt University the same year. As a senior, she did an independent research project on ecophysiology of forest herbs under the direction of Dr. William Eickmeier. In spring of 1992, she obtained her bachelor of science degree in Biology from Vanderbilt University. In fall of 1992, she entered the department of Botany at Louisiana State University and began her doctoral work under the direction of Dr. David Longstreth. She will receive her doctor of philosophy degree in December, 1997.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Elisabeth Ellen Schussler

Major Field: Plant Biology

Title of Dissertation: Aerenchyma Development in the Freshwater Marsh Species Sagittaria lancifolia L.

Approved:

David J. Ingestad
Major Professor and Chairman

John W. Darrin
Dean of the Graduate School

EXAMINING COMMITTEE:

Mary E. Musgrave

J. V. M.

Irving A. Mendelssohn

David H. Fickle

Date of Examination:

October 27, 1977

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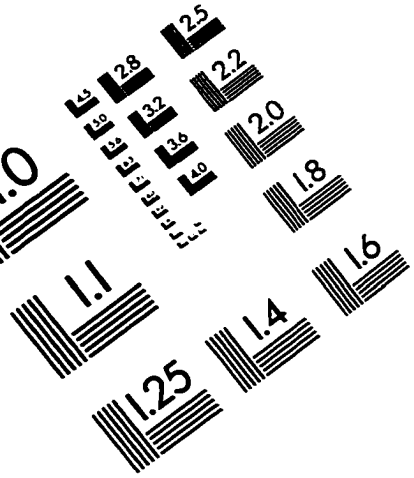
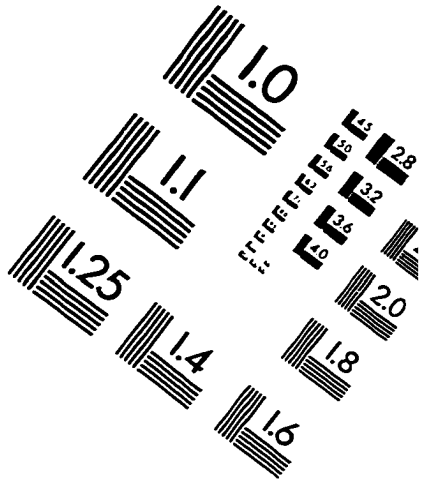
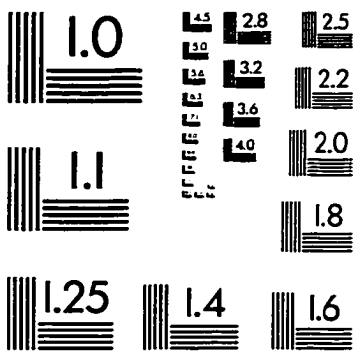
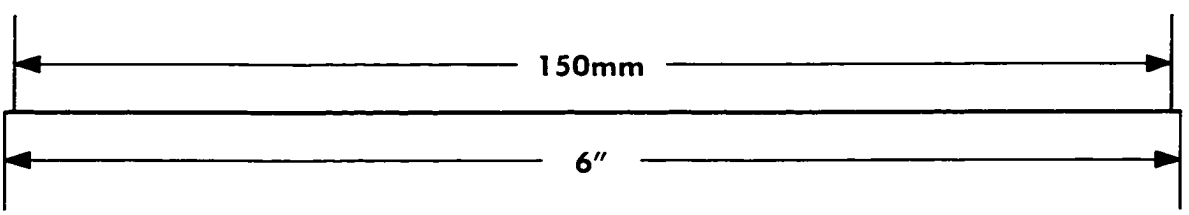
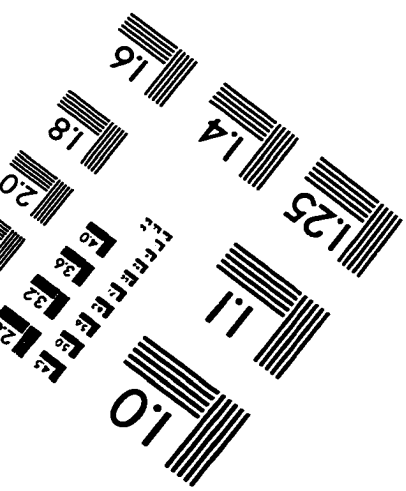
TEST TARGET (QA-3)

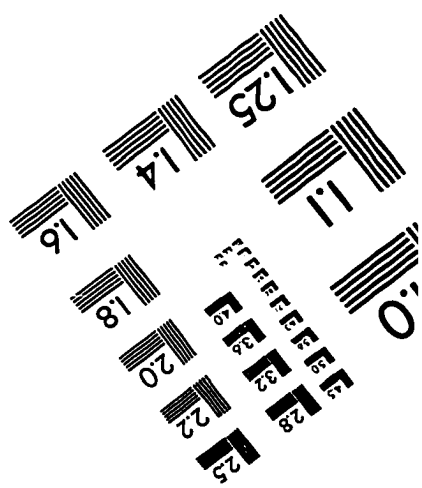












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